

HISTIDINE PROLINE RICH GLYCOPROTEIN (HPRG) AS AN ANTI-ANGIOGENIC AND ANTI-TUMOR AGENT

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention in the field of biochemistry and medicine is directed to novel methods for inhibiting angiogenesis and treating tumors and cancer using a glycoprotein termed "histidine proline rich glycoprotein" or biologically active fragments and other derivatives thereof.

Description of the Background Art

10 Angiogenesis, the formation of new capillaries from pre-existing ones (Folkman, J., *N. Engl. J. Med.*, 1971, 285:1182-1186; Hanahan D. *et al.*, *Cell*, 1996, 86:353-364), is a normal part of embryonic development, wound healing and female reproductive function. However, angiogenesis also plays a pathogenic role in the establishment and progression of certain diseases. Cancer, rheumatoid arthritis and diabetic retinopathy are examples of such diseases (Carmeliet P. *et al.*,
15 *Nature*, 2000, 407:249-257). Anti-angiogenic therapy holds promise in inhibiting the progression of these diseases.

Angiogenesis can be triggered by several pro-angiogenic cytokines. In the setting of cancer, tumor cells under hypoxic conditions secrete vascular endothelial growth factor (VEGF) and/or fibroblast growth factor (bFGF). These proteins diffuse and bind to specific receptors on
20 endothelial cells (ECs) in the local vasculature, perturbing the balance of pro- and anti-angiogenic forces in favor of angiogenesis. As a consequence of binding these proteins, ECs are activated to (a) secrete enzymes that induce remodeling of the associated tissue matrix, and (b) change the patterns and levels of expression of adhesion molecules such as integrins. Following matrix degradation, ECs proliferate and migrate toward the hypoxic tumor, resulting in the generation and
25 maturation of new blood vessels.

Interestingly, many anti-angiogenic factors result from the degradation of matrix proteins – *i.e.*, are a result of the action of pro-angiogenic enzymes. Examples include endostatin, a fragment of collagen XIII (O'Reilly, M. S. *et al.*, *Cell* 1997, 88:277-285); kringle 5 of plasminogen (O'Reilly, M. S. *et al.*, *Cell*, 994, 79:315-328) and PEX, the C-terminus non-catalytic subunit of
30 MMP-2 (Brooks P.C. *et al.*, *Cell*, 1998, 92:391-400).

The concept has emerged that, due to the abundance of pro-angiogenic factors, these anti-angiogenic molecules are unable to overcome the pro-angiogenic balance in a primary tumor. However, since they are secreted into circulation, these anti-angiogenic molecules are capable of inhibiting angiogenesis at other locations where tumor cells may have begun to invade.

Consequently, micro-metastases comprising these tumor cells at these new locations remain dormant. This hypothesis explains the puzzling observation made by surgeons many years ago: at various times after surgical removal of a primary tumor in a patient with no obvious metastatic disease, the patient returns with advanced metastatic disease.

Thus, clinical intervention by treatment with one or more of the anti-angiogenic factors could inhibit the angiogenic process and halt tumor growth as well as metastasis. Significant evidence in the literature (cited above) supports this notion.

Histidine Proline Rich Glycoprotein (HPRG = Histidine Rich Glycoprotein, HRG)

HPRG is synthesized in the liver (Morgan W.T., "Histidine-Rich Glycoprotein," In: *Encyclopedia of Molecular Medicine*, 2001. This glycoprotein has an unusually high percentage of Pro and His residues (human HPRG has 525 residues, 66 are His and 65 are Pro) which is reflected in its name. HPRG contains two cystatin-like domains at the N-terminus, and a His-Pro rich domain - also referred to herein as the "H/P domain" - (148 residues in human HPRG, of which 42 are His and 31 are Pro) between two Pro-rich domains at the C-terminus. The C-terminal domain is tethered back to the N-terminal domain (as in kininogen) and contains all three N-linked oligosaccharides; its sequence has diverged from cystatin enough to have lost all of the protease inhibitor activity of cystatin. HPRG is quite abundant in plasma (1.5 μ M, 125 μ g/ml). Despite this, very little is known about the physiological roles of HPRG.

HPRG binds a large array of ligands that can be divided in three major groups:

- (1) ligands belonging to the coagulation/fibrinolysis systems such as heparin, plasminogen, fibrinogen, vitronectin and thrombospondin;
- (2) small ligands, such as heme and transition metal ions (zinc, copper and nickel), and
- (3) cells such as T cells (Lamb-Wharton R.J. *et al.*, *Cellular Immunol.* 1993, 152:544-555; Olsen, HM *et al.*, *Immunology* 1996, 88:198-206), macrophages and platelets.

Based on the foregoing, several hypotheses have been proposed for possible roles for HPRG in modulating coagulation and fibrinolysis, metal transport and regulation of the immune system.

However, no hypothesis has yet been able to explain and integrate the apparent “promiscuity” of binding of this multidomain protein.

Some of the biological properties of HPRG depend on pH or metal binding. For instance, HPRG binding to heparin or to glycosaminoglycans (GAG) on the surface of ECs is dependent on low pH or abundant Zn^{+2} or Cu^{+2} (Borza D-B. *et al.*, *J. Biol. Chem.*, 1998, 273:5493-5499). Binding of Zn^{+2} or Cu^{+2} to the His-Pro-rich domain allows for subsequent binding to GAGs. Modest changes in pH of 0.25-0.50 units (from pH 7.4 of normal plasma), as may occur during hypoxia or ischemia, induce the protonation of the His residues of the H/P domain. Thus, pH and metal binding are exquisite regulators of HPRG activity.

HPRG binds plasminogen when in solution or when bound to GAG on the EC surface. This cell surface binding promotes activation of plasminogen to plasmin by tissue plasminogen activator (tPA) (Borza D-B. *et al.*, *J. Biol. Chem.*, 1997, 272:5718-5726), which is pro-angiogenic. The conserved C-terminal Lys is essential for the interaction with plasminogen as is the N-terminal domain. HPRG also binds to the γ -chains of fibrinogen. At pH 6.8, but not at pH 7.4, HPRG enhances polymerization of fibrin by thrombin.

Binding of chicken HPRG (cHPRG) to heparan sulfate proteoglycans has been shown to displace bFGF and α FGF from those sites (Brown K.J. *et al.*, *Biochemistry*, 1994, 33:13918).

While the effect of HPRG on angiogenesis has not been investigated, it was speculated that the abovementioned effect may promote or inhibit bFGF activity. Related to this property, cHPRG at concentrations of $\geq 80 \mu\text{g/ml}$ (approximately $1 \mu\text{M}$) significantly inhibited FGF-stimulated and baseline endogenous DNA synthesis in fibroblasts (Brown *et al. supra*). Since baseline proliferation was also inhibited, the effect may not be specific for FGF-stimulated DNA synthesis. Rather, HPRG may regulate DNA synthesis regardless of the nature of the stimulus. However, Brown *et al.* did not examine the possible effects of HPRG on ECs and angiogenic processes.

Rabbit and human HPRG are very similar in composition and function. Optimal alignment of the two proteins showed 63.5% sequence identity and 68.6% homology (Borza D-B. *et al.*, *Biochemistry*, 1996, 35:1925-1934). The highest homology is at the N- and C-termini. However, the apparent lower homology in the His-Pro rich domain is due to substitutions of Pro for His in the rabbit molecule. The human protein contains 15 repeats of the sequence HHPHG while the rabbit protein has 2 repeats of this sequence, 6 repeats of HPPHG and 7 repeats of PPPHG. Thus a consensus sequence for these repeating units is designated [H/P][H/P]PHG.

Simantov, R. *et al.*, *J. Clin. Invest.* 107:45-52 (2001) disclosed that HPRG inhibited the anti-angiogenic activity of thrombospondin (TSP-1) and concluded that regions of the HPRG are homologous to CD36, a TSP-1 receptor. These regions are at the N-terminus of HPRG, which contrasts from the present inventors' localization of anti-angiogenic activity to the H/P domain

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SUMMARY OF THE INVENTION

The present inventors have discovered that HPRG polypeptides or fragments thereof including domains and pentapeptides, altered conformations of HPRG, other biologically active derivatives of HPRG, exhibit anti-angiogenic and anti-tumor activity whereas antibodies specific for HPRG stimulate angiogenesis by blocking the action of HPRG *in vivo*. The anti-angiogenic action may occur in part through inhibition of oxidative stress, which has recently been demonstrated *in vitro* to contribute to the pathophysiology of angiogenesis (Brown *et al.* (2000) *Cancer Res.* 60:6298). Oxidative stress leading to angiogenesis may require transition metals such as zinc and copper-- small molecule copper chelators have been demonstrated to inhibit tumor growth *in vivo* (Brewer, GJ, International Patent publication WO/013712 (2000)).

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The present invention includes the first demonstration that proteinaceous metal chelator (HPRG) inhibits angiogenesis, possibly due to its binding transition metals. The present invention provides novel methods to inhibit or reduce angiogenesis, tumor growth, EC proliferation, EC migration or EC tube formation using HPRG, domains and peptide fragments, altered conformations and other biologically active derivatives.

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Transition metals and induction of oxidative stress have been implicated in the etiology of non-cancerous diseases, especially, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). Thus, the present invention also provides compositions and methods for the treatment of any disease whose pathobiology involves abnormal presence or undesired action of transition metals, including conditions where the presence of the transition metal may induce oxidative stress.

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The present invention provides an isolated anti-angiogenic polypeptide or peptide having the sequence of

- (a) the histidine-proline-rich (H/P) domain of human histidine-proline rich glycoprotein (HPRG) (SEQ ID NO:5)

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- (b) the H/P domain of human rabbit HPRG (SEQ ID NO:6)
- (c) a sequence variant of SEQ ID NO:5 or SEQ ID NO:6 having substantially the same biologic activity of inhibiting angiogenesis, endothelial cell proliferation or endothelial tube formation in an *in vitro* or *in vivo* bioassay;
- 5 (d) a pentapeptide from the H/P domain having the sequence (His,Pro)-(His,Pro)-Pro-His-Gly (SEQ ID NO:7), or an addition variant thereof having an additional 1 to 4 amino acids selected from the group consisting of His, Pro or Gly added at the N- or C-terminus of the pentapeptide.

The isolated peptide above preferably has a sequence selected from the group consisting of
 10 His-His-Pro-His-Gly (SEQ ID NO:8), His-Pro-Pro-His-Gly (SEQ ID NO:9), or Pro-Pro-Pro-His-Gly (SEQ ID NO:10).

Also provided is a chemically synthesized peptide multimer comprising the above peptide or addition variant, which multimer is selected from the group consisting of:

- (a) a multimer having the formula P^1_n wherein
 - 15 (i) P^1 is the peptide or addition variant of claim 2, and
 - (ii) $n=2-8$,
- (b) a multimer having the formula $(P^1-X_m)_n-P^2$, wherein
 - (i) P^1 and P^2 are pentapeptides or addition variants according to claim,
 - (ii) P^1 and P^2 are the same or different peptides;
 - 20 (iii) X is C_1-C_5 alkyl, C_1-C_5 alkenyl, C_1-C_5 alkynyl, C_1-C_5 polyether containing up to 4 oxygen atoms,
 - (iv) $m = 0$ or 1 and
 - (v) $n = 1-7$

and wherein the peptide multimer has the biological activity of inhibiting angiogenesis, endothelial
 25 cell proliferation or endothelial tube formation in an *in vitro* or *in vivo* bioassay.

Another embodiment is a recombinantly produced peptide multimer comprising the above peptide or addition variant, which multimer has the formula $(P^1-Gly_z)_n-P^2$, wherein:

- (i) P^1 and P^2 are pentapeptides or addition variants according to claim 2,
- (ii) P^1 and P^2 are the same or different;
- 30 (iii) $z = 0-6$; and
- (iv) $n = 1-100$.

The present invention is also directed to a diagnostically or therapeutically labeled anti-angiogenic polypeptide, peptide or peptide multimer comprising:

- 5 (a) the polypeptide, peptide or peptide multimer above, which is diagnostically or therapeutically labeled;
- (b) a diagnostically or therapeutically labeled human HPRG protein (SEQ ID NO:1);
- (c) a diagnostically or therapeutically labeled rabbit HPRG protein (SEQ ID NO:3); or
- (d) a diagnostically or therapeutically labeled polypeptide that is a homologue of (b) or (c).,

Preferably, the diagnostically or therapeutically labeled polypeptide or peptide is selected from the group consisting of: (a) the H/P domain of human HPRG (SEQ ID NO:5); (b) the H/P domain of rabbit HPRG (SEQ ID NO:6); and (c) the peptide having the sequence SEQ ID NO:7 or the addition variant thereof.

A diagnostically useful HPRG-related composition comprises the diagnostically labeled protein, peptide or peptide multimer as above, and a diagnostically acceptable carrier.

In the above diagnostic composition the detectable label is preferably a radionuclide, a PET-
15 imageable agent, an MRI-imageable agent, a fluorescer, a fluorogen, a chromophore, a chromogen,
a phosphorescer, a chemiluminescer or a bioluminescer.

Preferred radionuclides include ^3H , ^{14}C , ^{35}S , ^{67}Ga , ^{68}Ga , ^{72}As , ^{89}Zr , ^{97}Ru , ^{99}Tc , ^{111}In , ^{123}I , ^{125}I , ^{131}I , ^{169}Yb and ^{201}Tl .

Preferred fluorsceners or fluorogens include fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, fluorescamine, a fluorescein derivative, Oregon Green, Rhodamine Green, Rhodol Green and Texas Red.

An anti-angiogenic pharmaceutical composition comprises an effective amount of the protein peptide or peptide multimer of any of claims 1-4; and a pharmaceutically acceptable carrier.

In one embodiment, a therapeutic anti-angiogenic pharmaceutical composition comprises an effective amount of the polypeptide, peptide or peptide multimer described above to which is bound directly or indirectly a therapeutically active moiety; and a pharmaceutically acceptable carrier. Preferably the pharmaceutical composition is in a form suitable for injection.

The therapeutically active moiety may be a radionuclide, preferably ^{47}Sc , ^{67}Cu , ^{90}Y , ^{109}Pd , ^{125}I , ^{131}I , ^{186}Re , ^{188}Re , ^{199}Au , ^{211}At , ^{212}Pb or ^{217}Bi .

30 This invention is also directed to an antibody specific for an epitope of HPRG that is present in the H/P domain of human HPRG (SEQ ID NO:5) or the H/P domain of rabbit HPRG (SEQ ID

NO:6), and which binds to HPRG or to any of the domains in a way which inhibits the anti-angiogenic activity of HPRG or the domain, (or an antigen-binding fragment of the antibody). The epitope recognized by the antibody or fragment preferably comprises a pentapeptide from the H/P domain having the sequence His-His-Pro-His-Gly (SEQ ID NO:8), His-Pro-Pro-His-Gly (SEQ ID NO:9), or Pro-Pro-Pro-His-Gly (SEQ ID NO:10). The antibody may be a monoclonal antibody, including a human or humanized monoclonal antibody.

An antibody embodiment useful for detecting HPRG comprises the above antibody or fragment which is detectably labeled.

A therapeutically useful antibody that targets HPRG or an epitope thereof comprises the above antibody or fragment to which is bound directly or indirectly a therapeutically active moiety.

The invention provides a pharmaceutical composition that stimulates angiogenesis *in vitro* or *in vivo*, comprising: (a) the antibody or fragment above; and (b) a pharmaceutically acceptable carrier.

This invention provides a method for inhibiting cell migration, cell invasion, cell proliferation or angiogenesis, or for inducing apoptosis, comprising contacting cells associated with undesired cell migration, invasion, proliferation or angiogenesis with an effective amount of a therapeutic pharmaceutical composition as described above.

Also included is a method for treating a subject having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, comprising administering to the subject an effective amount of the pharmaceutical composition comprising the polypeptide, peptide or multimer. A preferred disease or condition for this treatment is a tumor or cancer.

Another method for stimulating angiogenesis comprises providing to cells participating in angiogenesis an effective amount of the antibody or fragment above. A method for stimulating angiogenesis in a subject in need of enhanced angiogenesis comprises administering to the subject an effective amount of the above antibody-based pharmaceutical composition.

Also provides is a method for detecting the presence of HPRG or cleavage product or peptide thereof in a biological sample, comprising the steps of:

- (a) contacting the sample with the antibody or fragment of claim 20; and
- (b) detecting the presence of the label associated with the sample.

The sample is preferably plasma, serum, cells, a tissue, an organ, or an extract of the cells, tissue or organ. The contacting and the detecting may be *in vitro*; alternatively, the contacting is *in*

vivo and the detecting is *in vitro* or vice versa. In another embodiment, the contacting and the detecting are *in vivo*

The present invention is also directed to an isolated nucleic acid that encodes the polypeptide or peptide or peptide multimer described above. An expression vector of this invention comprises the above nucleic acid of claim operatively linked to a promoter and optionally, additional regulatory sequences that regulate expression of the nucleic acid in a eukaryotic cell. A preferred expression vector is a plasmid or a viral vector.

Also included is a cell transformed or transfected with the above nucleic acid molecule or expression vector. The cell is preferably a mammalian cell, most preferably a human cell.

The invention includes a method for providing to a cell, tissue or organ an angiogenesis-inhibitory amount of a HPRG, an H/P domain of HPRG or a pentapeptide of the H/P domain having the sequence (His,Pro)-(His,Pro)-Pro-His-Gly (SEQ ID NO:7), or a peptide multimer that includes the pentapeptide, comprising: administering to the cell tissue or organ, the above expression vector such that the nucleic acid is taken up and expressed in the cell, tissue or organ. The administering is preferably *in vivo*.

Also included is a method for providing to a cell, tissue or organ an angiogenesis-inhibitory amount of a HPRG, an H/P domain of HPRG, a pentapeptide of the H/P domain having the sequence (His,Pro)-(His,Pro)-Pro-His-Gly (SEQ ID NO:7), or a peptide multimer that includes the pentapeptide, comprising: contacting the cell tissue or organ, with the above transformed or transfected cells, wherein the administered cells express the polypeptide, peptide or peptide multimer. Preferably, the contacting is *in vivo*.

This invention is also directed to a method for inhibiting angiogenesis in a subject in need of such inhibition, comprising administering to the subject an effective amount of the expression vector as above, such that the nucleic acid is expressed resulting in the presence of an angiogenesis-inhibiting amount of the polypeptide, peptide or peptide multimer, thereby inhibiting the angiogenesis.

Another method for inhibiting angiogenesis in a subject in need of such inhibition, comprises administering to the subject an effective amount of the transformed or transfected cells as above, which cells produce and provide in the subject an angiogenesis-inhibiting amount of the polypeptide, peptide or peptide multimer, thereby inhibiting the angiogenesis.

In the above methods, the subject has a tumor, and the angiogenesis inhibition results in reduction in size or growth rate of the tumor or destruction of the tumor. Preferably, the subject is a human.

5 A longer example of a disease or condition against which the above method is effective include primary growth of a solid tumor, leukemia or lymphoma; tumor invasion, metastasis or growth of tumor metastases; benign hyperplasia; atherosclerosis; myocardial angiogenesis; post-balloon angioplasty vascular restenosis; neointima formation following vascular trauma; vascular graft restenosis; coronary collateral formation; deep venous thrombosis; ischemic limb angiogenesis; telangiectasia; pyogenic granuloma; corneal disease; rubeosis; neovascular glaucoma; 10 diabetic and other retinopathy; retrolental fibroplasia; diabetic neovascularization; macular degeneration; endometriosis; arthritis; fibrosis associated with a chronic inflammatory condition, traumatic spinal cord injury including ischemia, scarring or fibrosis; lung fibrosis, chemotherapy-induced fibrosis; wound healing with scarring and fibrosis; peptic ulcers; a bone fracture; keloids; or a disorder of vasculogenesis, hematopoiesis, ovulation, menstruation, pregnancy or placentation 15 associated with pathogenic cell invasion or with angiogenesis.

A preferred disease or condition to be treated by the above method is tumor growth, invasion or metastasis. This includes brain tumors. Examples of such brain tumors are astrocytoma, anaplastic astrocytoma, glioblastoma, glioblastoma multiformae, pilocytic astrocytoma, pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma, fibrillary 20 astrocytoma, gemistocytic astrocytoma, protoplasmic astrocytoma, oligodendroglioma, anaplastic oligodendroglioma, ependymoma, anaplastic ependymoma, myxopapillary ependymoma, subependymoma, mixed oligoastrocytoma and malignant oligoastrocytoma..

The method is also used to treat a uterine disease such as endometriosis and pathogenic ocular neovascularization such as that associated with, or a cause of, proliferative diabetic 25 retinopathy, neovascular age-related macular degeneration, retinopathy of prematurity, sickle cell retinopathy or retinal vein occlusion.

Also provided herein is an "HPRG affinity ligand" useful for binding to or isolating HPRG-ligands, binding sites or cells expressing the ligands or binding sites, comprising the above polypeptides, peptide or peptide multimers immobilized to a solid support or carrier.

This affinity ligand is used in a method for isolating a HPRG protein or peptide from a complex mixture comprising:

- (a) contacting the mixture with the affinity ligand above;
- (b) allowing any material in the mixture to bind to said ligand;
- 5 (c) removing unbound material from said ligand; and
- (d) eluting the bound HPRG protein or peptide.

Also provided is a method for isolating or enriching cells expressing a HPRG binding site/receptor from a cell mixture, comprising

- (a) contacting said cell mixture with the above HPRG affinity ligand;
- 10 (b) allowing any cells expressing the binding site to bind to said compound;
- (c) separating cells bound to said compound from unbound cells; and
- (d) removing said bound cells,

thereby isolating or enriching said HPRG binding site-expressing cells.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a schematic diagram of the structure of HPRG, showing the various domains. The scissors indicate the position of plasmin cleavage sites.

Figure 2A and 2B show inhibition of bFGF -stimulated proliferation of human umbilical vein endothelial cells (HUVEC). Rabbit HPRG (Fig. 2A) and its His-Pro rich ("H/P") domain inhibit proliferation of HUVEC

20 Figure 3 shows the induction of caspase-3 in bFGF-stimulated HUVEC by HPRG and HKa, the two-chain human kininogen protein.

Figure 4A and 4B are photomicrographs of HUVEC plated on Matrigel®-coated 96 well plates showing the inhibition of EC tube formation by HPRG (Fig. 4B) compared to a control (Fig. 4A).

25 Figure 5 shows the inhibition of angiogenesis in the chorioallantoic membrane (CAM) using chick embryos. HPRG (ATN-234) and the H/P domain (ATN-236) are shown to inhibit angiogenesis, expressed as blood vessel number.

Figure 6 shows that HPRG and the H/P domain inhibit angiogenesis stimulated by FGF-2 in Matrigel® plug model *in vivo*.

Figure 7 shows that HPRG and the H/P domain inhibit 3LL tumor-mediated angiogenesis in Matrigel® plug model *in vivo*.

Figure 8A and 8B show that the H/P domain of HPRG inhibits growth of (Fig. 8A) and angiogenesis by (Fig. 8B) MatLyLu tumor cells *in vivo* in a Matrigel® Plug model. The H/P domain was tested at 1.8 μM (as was the positive control endostatin protein).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

No role for HPRG as an inhibitor of angiogenesis had been suggested prior to the making of the present invention. The present inventors conceived that native HPRG and biologically active HPRG polypeptides, homologues, variants and other functional derivatives including peptide fragments and conformers of HPRG, as well as antibodies specific for HPRG exhibit anti-angiogenic activity and, therefore, anti-tumor activity. Pharmaceutical compositions comprising these compounds are useful in the treatment of cancer and other diseases associated with aberrant or undesired angiogenesis.

Human HPRG has the amino acid sequence SEQ ID NO:1:

15	10	20	30	40	50	60
	MKALIAALLL	ITLQYSCAVS	PTDCSAVEPE	AEKALDLINK	RRRDGYLFQL	LRIADAHLDR
	70	80	90	100	110	120
20	VENTTVYYLV	LDVQESDCSV	LSRKYWNDCE	PPDSRRPSEI	VIGQCKVIAT	RHSHESQDLR
	130	140	150	160	170	180
	VIDFNCTTSS	VSSALANTKD	SPVLIDFFED	TERYRKQANK	ALEKYKEEND	DFASFRVDRI
	190	200	210	220	230	240
25	ERVARVRGGE	GTGYFVDFSV	RNCPRHHFPR	HPNVFGFCRA	DLFYDVEALD	LESPKNLVIN
	250	260	270	280	290	300
	CEVFDPQEHE	NINGVPPHLG	HPFHWGGHER	<u>SSTTKPPEKP</u>	<u>HGSRDHHHPH</u>	<u>KPHEHGPPPP</u>
	310	320	330	340	350	360
30	<u>PDERDHSHPG</u>	<u>PLPQGPPPLL</u>	<u>PMSCSSCQHA</u>	<u>TFGTNGAQRH</u>	<u>SHNNNSSDLH</u>	<u>PHKHHSHEQH</u>
	370	380	390	400	410	420
35	<u>PHGHHPHAHH</u>	<u>PHEHDTHRQH</u>	<u>PHGHHPHGHH</u>	<u>PHGHHPHGHH</u>	<u>PHGHHPHCHD</u>	<u>FQDYGPCDPP</u>
	430	440	450	460	470	480
	<u>PHNQGHCCHG</u>	<u>HGPPPGHLRR</u>	<u>RGPGKGPRPF</u>	<u>HCRQIGSVYR</u>	<u>LPPLRKGEVL</u>	<u>PLPEANFPSE</u>

490 500 510 520 525
| | | | |
PLPHHKHPLK PDNQFPQSV SESC PGKFKS GFPQVSMFFT HTFPK

italic: signal sequence

double underscore: Pro-rich domain

single underscore: His-Pro (H/P) rich domain

Thus, human HPRG consists of 525 amino acids residues, has a molecular mass of weight: 59,578

Da and a theoretical pI of 7.09

Human HPRG is encoded by the DNA of the following sequence (SEQ ID NO:2)

10	1	atataatata	aactaataaa	gatacagga	taattaatgt	ataccgtaat	gtagaccgac
	61	tcaggatatgt	aagtagagaa	tatgaagggt	aattagataa	ttaaagggat	ggtttaacaa
	121	aatgaaggca	ctcattgcag	cactgctttt	gatcacattg	cagtattcgt	gtgccgtgag
	181	tccccactgac	tgcagtgcgt	ttgagccgga	ggctgagaaa	gctctagacc	tgatcaacaa
	241	aaggcgacgg	gatggctacc	ttttccaatt	gctgcggtt	gctgatgcc	acttggacag
15	301	agtggaaaat	acaactgtat	attacttagt	cttagatgtg	caagaatcgg	actgttcggt
	361	cctatccagg	aaatactgga	atgactgtga	gccacctgat	tccagacgtc	catctgaaat
	421	agtgatcgga	caatgtaagg	taatagctac	aagacattcc	catgaatctc	aggacctcag
	481	agtgattgac	tttaactgca	ccacaagttc	tgtctcttca	gcactggcca	ataccaaaga
	541	tagtccggtc	ctcatagatt	tctttgagga	tactgagcgc	tacagaaaac	aagccaacaa
20	601	agcccttgag	aagtacaaag	aggagaatga	tgactttgcc	tctttcagag	tggaccgaat
	661	cgagagagtt	gcaagagtga	gaggagggga	aggaactggt	tacttcgtgg	acttctctgt
	721	gcggaactgc	cccagacacc	atttcccag	acaccccaat	gtctttggat	tctgcagagc
	781	agatttggtc	tatgatgtag	aagccttgga	cttggaagc	ccgaaaaacc	ttgtcataaa
	841	ctgtgaagtc	ttcgaccctc	aggaacatga	gaacatcaat	ggtgtaccgc	ctcattttggg
25	901	acatcccctc	cactgggggtg	ggcatgagcg	ttcttctacc	accaagcctc	cattcaagcc
	961	ccatggatct	agagatcatc	atcatcccca	caagccacac	gaacatggac	ccccacctcc
	1021	tccagatgaa	agagatcact	cacatggacc	cccacttcca	caaggccctc	ctccactatt
	1081	gccccatgtcc	tgctcaagtt	gtcaacatgc	cacttttggc	acaaatgggg	cccaaagaca
	1141	ttctcataat	aataattcca	gtgacctcca	tccccataag	catcattccc	atgaacagca
30	1201	tccccacgga	caccatcccc	atgcacacca	tcctcatgaa	catgataccc	atagacagca
	1261	tccccatgga	caccaccccc	atggacacca	tcctcatgga	caccaccccc	atggacacca
	1321	tccccatgga	caccatcccc	actgccatga	tttccaagac	tatggacctt	gtgaccacc
	1381	accccataac	caaggtcact	gttgccatgg	ccacggccca	ccacctgggc	acttaagaag
	1441	gcgaggccca	ggtaaaaggac	cccgctccctt	ccattgcaga	caaattggat	ctgtgtaccg
35	1501	actccctcct	ctaagaaaag	gtgaggtgct	gccacttcct	gaggccaatt	ttcccagctt
	1561	cccattgccg	caccacaaac	atcctctaaa	gccagacaat	cagccctttc	ctcaatcagt
	1621	ctctgaatca	tgtccaggga	agttcaagag	tgggtttcca	caagtttcca	tgttttttac
	1681	acatacatctt	ccaaaataaa	atgtgattcc	tttgaagagg	aaaatgaata	atacattgaa
	1741	ttagaaacat	aaataaaaatg	accagtaatt	gtgaaaatta	cagttctttt	caacctactt
40	1801	tcatactgaa	gatgcagcaa	aatgtgaatg	ggaaaagaga	tggcctgaga	agagagatca
	1861	aatggaaagg	agaggaaaaga	actcagtgtc	gcctattagt	agttaattct	gtcactcacc
	1921	actacatcac	ttgagacaaa	tctatgccac	tcagaatctc	cttctttcct	ggacttaact
	1981	ctaattctag	agtctctgtt	actgcttggg	ctatacctgg	gcataactaat	aaagtatggt
	2041	attgaaacta	t	2051			

Rabbit HPRG has the amino acid sequence SEQ ID NO:3 as follows:

10 20 30 40 50 60
| | | | |
ATLQCSWALT PTDCCKTTKPL AEKALDLINK WRRDGYLFQL LRVADAHLDG AESATVYYLV

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      70      80      90      100      110      120
LDVKETDCSV LSRKHWEDCD PDLTKRPSLD VIGQCKVIAT RYSDEYQTLR LNDFNCTTSS
      130      140      150      160      170      180
5  VSSALANTKD SPVLFDIED TEPFRKSADK ALEVYKSESE AYASFRVDRV ERVTRVKGGE
      190      200      210      220      230      240
RTNYYVDFSV RNCSSRSFHR HPAFGFCRAD LSFDEASNL ENPEDVIISC EVFNFEHGN
10      250      260      270      280      290      300
ISGFRPHLGK TPLGTDGSRD HHHPHKPHKE GCPPPQEGED FSEGPPLQGG TPPLSPFRP
      310      320      330      340      350      360
15 RCRHRPFGTN ETHRFPHHRI SVNIIHRPPP HGHHPHGPPP HGHHPHGPPP HGHPHGPPP
      370      380      390      400      410      420
RHPPHGPPPH GHPPHGPPPH GHPPHGPPPH GHPPHGPPPH GHPPHGPHGFH DHGPCDPPSH
      430      440      450      460      470      480
20 KEGPQDLHQH AMGPPPKHPG KRGPGKGHFP FHWRRIGSVY QLPPLQKGEV LPLPEANFPQ
      490      500      510      520      526
LLLRNHTHPL KPEIQPFQV ASERCPEEFN GEFAQLSKFF PSTFPK

```

italic: signal sequence

double underscore: Pro-rich domain

single underscore: His-Pro (H/P) rich domain

The rabbit protein is encoded by a DNA molecule having the sequence: SEQ ID NO:4

```

1  gcgccacact gcagtgttcg tgggctttga ctccactga ctgcaaaaact accaagccct
30  61  tggctgagaa agctctagac ctgatcaata aatggcgacg ggatggctac cttttccagt
121 tgctgcgagt cgctgatgcc cacttggacg gagcggaaatc tgccactgtc tactatttag
181 tcttagatgt gaaagagact gactgttcag tgctatccag gaaacactgg gaagactgtg
241 acccagatct tactaaacgt ccatctcttg acgtgattgg gcaatgtaag gtgatagcta
301 ccagatatcc ggatgaatat cagactctaa gattgaatga ctttaactgc accacgagtt
35  361 ccgtctcttc agccctggcc aacactaaag acagtcctgt tctctttgat ttcacgagg
421 acacggagcc cttcagaaaa tccgcggaca aagccctgga ggtgtacaaa agtgaaagcg
481 aggcgtatgc ctctttcaga gtggaccggg tagagagagt cacaaggggtg aaaggaggag
541 agagaaccaa ttactatgtg gacttctccg tgaggaactg ctccaggtct cacttccaca
601 gacaccccg ctttgggttc tgcagagcag atctgtcctt tgatgtagaa gcctcgaact
40  661 tggaaaaccc agaagacgtt attataagct gtgaagtctt taactttgag gaacatggaa
721 acatcagtgg ttttcgaccc catttgggca agactccact tgggactgat ggatccagag
781 atcatcatca tccccacaag ccacataagt ttggatgccc acctcccca gaaggggaag
841 atttctcgga aggaccacca cttcaagggtg gaaccccccc actctcccc cccttcaggc
901 caagatgtcg tcatcgccct tttggcacca atgaaaccca tcggttcctt catcatcgaa
45  961 tttcagtga catcatccat agggcccttc cccatggaca tcaccccat gggcccttc
1021 cccatggaca tcaccccat gggcccttc cccatggaca tcctcctcat ggaccccttc
1081 cccgacatcc tccccatggg cctcctcccc atggacatcc ccccatgga cccctcccc
1141 atggacatcc tcctcatgga cccctcccc atggacatcc tccccatggg cccctcccc
1201 atggacatcc tccccatggc catggtttcc atgacatgg accctgtgac ccaccatccc
50  1261 ataaagaagg tccccagac ctccatcagc atgcatggg accaccacct aagcaccag
1321 gaaagagagg tccaggtaaa ggacactttc cttccactg gagaagaatt gggctctgtt

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5 1381 accaactgcc cccactgcag aaaggtgaag tccttcccct tcccgaagcc aattttcccc
1441 agcttctctt gcggaaccac acccaccctc taaagcccga gatccagccc ttccctcagg
1501 tagcctctga gcgctgtcca gaggagtcca atggtgagtt tgcacaactc tccaagtttt
1561 tcccatctac atttccaaaa tgaaatctga tttccttgat gggnaacaat gaatgatatt
1621 ctgtattagc accataaata aaatgtggcc atgatgaatg ca

Preferred polypeptides are the H/P domain of human HPRG,

10 HPHKHHSHEQ HPHGHHPHAH HPHEHDTHRQ HPHGHHPHGH HPHGHHPHGH HPHGHHPHCH
DFQDYGPCDP PPHNQGHCH GHGPPPGHLR RRGPGKGPRP FHCRIQISVY RLPPLRKGEV
LPLPEANFPS FPLPHHKHPL KPDNQPPF (SEQ ID NO:5)

and the H/P domain of rabbit HPRG,

15 SVNIIHRPPP HGHHHPGPPP HGHHHPGPPP HGHPHPGPPP RHPHPGPPPH GHPPHPGPPH
GHPPHPGPPH GHPPHPGPPH GHPPHGHGFH DHGPCDPPSHK (SEQ ID NO:6)

Further, homologues of the HPRG protein or of its domains (*e.g.*, Borza *et al.*, 1996. *supra*) or peptides thereof that share sequence similarity with HPRG also exhibit anti-angiogenic and anti-tumor activity.

20 Examples of such homologues are *Plasmodium falciparum* erythrocyte membrane protein-1, *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and the histatin family of proteins.

25 A functional homologue must possess the biochemical and biological activity, preferably anti-angiogenic and anti-tumor activity which can be tested using *in vitro* or *in vivo* methods described herein. In view of this functional characterization, use of homologous HPRG proteins from other species, including proteins not yet discovered, falls within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

30 To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

35 In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. For example, preferred alignment would be with human HPRG protein H/P domain (SEQ ID NO:5) or rabbit HPRG protein H/P domain (SEQ ID NO:6), at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60% and even more preferably at least 70, 80 or 90 % of the amino acid residues are aligned. The amino acid residues (or nucleotides from the coding

sequence) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to human or murine HPRG nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HPRG protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs,

the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>

Thus, a homologue of the HPRG described above is characterized as having (a) functional activity of native HPRG, and (b) sequence similarity to a native HPRG when determined above, of at least about 30% (at the amino acid level), preferably at least about 50%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of HPRG. Then, the protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein. A biological assay of endothelial cell proliferation will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

Peptide Compositions

A preferred composition is, or comprises, a biologically active peptide of HPRG characterized in that it possesses the binding and/or biological activity of HPRG. Such binding is to a ligand that is preferably a member of the following ligand classes:

- (1) ligands belonging to the coagulation/fibrinolysis systems such as heparin, plasminogen, fibrinogen, vitronectin and thrombospondin. HPRG may bind similarly to other molecules that interact with these ligands. Thus, the present invention preferably includes novel any molecule that binds to the above-mentioned ligands.
- (2) small ligands, such as heme or transition metal ions (zinc, copper and nickel), or
- (3) cells such as T cells, macrophages and platelets.

Moreover, a biologically active peptide has HPRG activity in an *in vitro* or *in vivo* assay of binding or of biological activity such as those characterized herein. Preferably the peptide inhibits endothelial cell proliferation or migration, EC tube formation, angiogenesis or tumor growth at a level at least about 20 % of the activity of full length HPRG.

A preferred peptide comprises a minimal consensus sequence [H/P][H/P]PHG (SEQ ID NO:7) that is derived from the comparison of the amino acid sequence of one or more domains of HPRG among different species. An addition variant of such a consensus sequence peptide has between 1-4 additional amino acids selected from H, P and G in any combination. Longer peptide multimers of the invention are described below.

The peptide may be capped at its N and C termini with an acyl (abbreviated "Ac") -and an amido (abbreviated "Am") group, respectively, for example acetyl (CH₃CO-) at the N terminus and amido (-NH₂) at the C terminus.

A broad range of N-terminal capping functions, preferably in a linkage to the terminal amino group, is contemplated, for example:

formyl;

alkanoyl, having from 1 to 10 carbon atoms, such as acetyl, propionyl, butyryl;

alkenoyl, having from 1 to 10 carbon atoms, such as hex-3-enoyl;

alkynoyl, having from 1 to 10 carbon atoms, such as hex-5-ynoyl;

aroyl, such as benzoyl or 1-naphthoyl;

heteroaroyl, such as 3-pyrrolyl or 4-quinoloyl;

alkylsulfonyl, such as methanesulfonyl;

arylsulfonyl, such as benzenesulfonyl or sulfanilyl;

heteroarylsulfonyl, such as pyridine-4-sulfonyl;

substituted alkanoyl, having from 1 to 10 carbon atoms, such as 4-aminobutyryl;

substituted alkenoyl, having from 1 to 10 carbon atoms, such as 6-hydroxy-hex-3-enoyl;

substituted alkynoyl, having from 1 to 10 carbon atoms, such as 3-hydroxy-hex-5-ynoyl;

substituted aroyl, such as 4-chlorobenzoyl or 8-hydroxy-naphth-2-oyl;

substituted heteroaroyl, such as 2,4-dioxo-1,2,3,4-tetrahydro-3-methyl-quinazolin-6-oyl;

substituted alkylsulfonyl, such as 2-aminoethanesulfonyl;

substituted arylsulfonyl, such as 5-dimethylamino-1-naphthalenesulfonyl;

substituted heteroarylsulfonyl, such as 1-methoxy-6-isoquinolinesulfonyl;

carbamoyl or thiocarbamoyl;

substituted carbamoyl (R'-NH-CO) or substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkyl, alkenyl, alkynyl, aryl, heteroaryl, substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, or substituted heteroaryl;

substituted carbamoyl (R'-NH-CO) and substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkanoyl, alkenoyl, alkynoyl, aroyl, heteroaroyl, substituted alkanoyl, substituted alkenoyl, substituted alkynoyl, substituted aroyl, or substituted heteroaroyl, all as above defined.

The C-terminal capping function can either be in an amide or ester bond with the terminal carboxyl. Capping functions that provide for an amide bond are designated as NR¹R² wherein R¹ and R² may be independently drawn from the following group:

hydrogen;

alkyl, preferably having from 1 to 10 carbon atoms, such as methyl, ethyl, isopropyl;

alkenyl, preferably having from 1 to 10 carbon atoms, such as prop-2-enyl;

alkynyl, preferably having from 1 to 10 carbon atoms, such as prop-2-ynyl;

substituted alkyl having from 1 to 10 carbon atoms, such as hydroxyalkyl, alkoxyalkyl, mercaptoalkyl, alkylthioalkyl, halogenoalkyl, cyanoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkanoylalkyl, carboxyalkyl, carbamoylalkyl;

substituted alkenyl having from 1 to 10 carbon atoms, such as hydroxyalkenyl, alkoxyalkenyl, mercaptoalkenyl, alkylthioalkenyl, halogenoalkenyl, cyanoalkenyl, aminoalkenyl, alkylaminoalkenyl, dialkylaminoalkenyl, alkanoylalkenyl, carboxyalkenyl, carbamoylalkenyl;

substituted alkynyl having from 1 to 10 carbon atoms, such as hydroxyalkynyl, alkoxyalkynyl, mercaptoalkynyl, alkylthioalkynyl, halogenoalkynyl, cyanoalkynyl, aminoalkynyl, alkylaminoalkynyl, dialkylaminoalkynyl, alkanoylalkynyl, carboxyalkynyl, carbamoylalkynyl;

aroylalkyl having up to 10 carbon atoms, such as phenacyl or 2-benzoyl ethyl;

aryl, such as phenyl or 1-naphthyl;

heteroaryl, such as 4-quinolyl;

alkanoyl having from 1 to 10 carbon atoms, such as acetyl or butyryl;

aroyl, such as benzoyl;

heteroaroyl, such as 3-quinoloyl;

OR' or NR'R'' where R' and R'' are independently hydrogen, alkyl, aryl, heteroaryl, acyl, aroyl, sulfonyl, sulfinyl, or SO₂-R''' or SO-R''' where R''' is substituted or unsubstituted alkyl, aryl, heteroaryl, alkenyl, or alkynyl.

Capping functions that provide for an ester bond are designated as OR, wherein R may be: alkoxy; aryloxy; heteroaryloxy; aralkyloxy; heteroaralkyloxy; substituted alkoxy; substituted aryloxy; substituted heteroaryloxy; substituted aralkyloxy; or substituted heteroaralkyloxy.

Either the N-terminal or the C-terminal capping function, or both, may be of such structure that the capped molecule functions as a prodrug (a pharmacologically inactive derivative of the parent drug molecule) that undergoes spontaneous or enzymatic transformation within the body in

order to release the active drug and that has improved delivery properties over the parent drug molecule (Bundgaard H, Ed: *Design of Prodrugs*, Elsevier, Amsterdam, 1985).

Judicious choice of capping groups allows the addition of other activities on the peptide. For example, the presence of a sulfhydryl group linked to the N- or C-terminal cap will permit conjugation of the derivatized peptide to other molecules.

Production of Peptides and Derivatives

General Chemical Synthetic Procedures

The peptides of the invention may be prepared using recombinant DNA technology. However, given their length, they are preferably prepared using solid-phase synthesis, such as that generally described by Merrifield, *J. Amer. Chem. Soc.*, 85:2149-54 (1963), although other equivalent chemical syntheses known in the art are also useful. Solid-phase peptide synthesis may be initiated from the C-terminus of the peptide by coupling a protected α -amino acid to a suitable resin. Such a starting material can be prepared by attaching an α -amino-protected amino acid by an ester linkage to a chloromethylated resin or to a hydroxymethyl resin, or by an amide bond to a BHA resin or MBHA resin.

Such methods, well-known in the art, are disclosed, for example, in U.S. 5,994,309 (issued 11/30/1999) which is incorporated by reference in its entirety.

Amino Acid Substitution and Addition Variants

Also included in this invention are peptides in which at least one amino acid residue and preferably, only one, has been removed and a different residue inserted in its place compared to the native sequence. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1979, and Creighton, T.E., *Proteins: Structure and Molecular Principles*, W.H. Freeman & Co., San Francisco, 1984, which are hereby incorporated by reference. The types of substitutions which may be made in the peptide molecule of the present invention are conservative substitutions and are defined herein as exchanges within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: *e.g.*, Ala, Ser, Thr, Gly;
2. Polar, negatively charged residues and their amides: *e.g.*, Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: *e.g.*, His, Arg, Lys;

Pro, because of its unusual geometry, tightly constrains the chain. Substantial changes in functional properties are made by selecting substitutions that are less conservative, such as between, rather

than within, the above groups (or two other amino acid groups not shown above), which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Most substitutions according to the present invention are those that do not
5 produce radical changes in the characteristics of the peptide molecule. Even when it is difficult to predict the exact effect of a substitution in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays, preferably the biological assays described below. Modifications of peptide properties including redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers
10 or into multimers are assayed by methods well known to the ordinarily skilled artisan.

The present invention provides methods to inhibit or reduce angiogenesis, tumor growth, EC proliferation, EC migration or EC tube formation.

The invention also provides pharmaceutical compositions comprising fragments, peptides, conformers, antibodies, biological equivalents or derivatives of HPRG.

15 The HPRG used in the present invention can be derived from any organism that produces it in nature such as rabbits or, preferably, humans. The nucleotide sequence (SEQ ID NO:2 and amino acid sequence (SEQ ID NO:1) of human HPRG are available from GenBank (GenBank Accession number M1349, and Swiss Prot number: PO4196).

20 HPRG is isolated from a body fluid such as blood and urine, though it can also be obtained from other sources such as tissue extracts or as a product of a cell line growing in culture that produces "native" HPRG or that has been genetically modified with DNA encoding native HPRG or a functional derivative thereof to express this protein or a functional derivative thereof such as a domain or shorter fragment.

25 HPRG, fragments or derivatives are chemically synthesized, or produced by recombinant methods. Recombinant techniques known in the art include, but are not limited to DNA amplification using PCR of a cDNA library for example by reverse transcription of mRNA in cells extracts followed by PCR.

30 Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, F.M. *et al.* *Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene*

5 *Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press,
Berkeley, CA (1981).

10 angiogenesis, EC proliferation, migration or tube formation and/or tumor growth.

Chemical Derivatives of HPRG

15 and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein.

20 Cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding
amines) to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are
derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazolyl) propionic acid,
chloroacetyl phosphate, N- alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide,
p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-
25 diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate (pH 5.5-7.0) which agent is relatively specific for the histidyl side chain. *p*-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

30 Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include

imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, including phenylglyoxal, 2,3- butanedione, 1,2-cyclohexanedione, and ninhydrin. Such derivatization requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine ϵ -amino group.

Modification of tyrosyl residues has permits introduction of spectral labels into a peptide. This is accomplished by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to create O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides ($R-N=C=N-R'$) such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. Conversely, glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues. Deamidation can be performed under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or other macromolecular carrier. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'- dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane.

Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propionimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates

and the reactive substrates described in U.S. Patents 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of the hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecule Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Also included are peptides wherein one or more D-amino acids are substituted for one or more L-amino acids.

Multimeric Peptides

The present invention also includes longer peptides built from repeating units of one or more sequences from the H/P domain of the HPRG protein that have anti-angiogenic activity. The preferred peptide unit of such a multimer is a pentapeptide, preferably His-His-Pro-His-Gly (SEQ ID NO:8), His-Pro-Pro-His-Gly (SEQ ID NO:9), or Pro-Pro-Pro-His-Gly (SEQ ID NO:10).

Addition variants of these peptide units preferably include from 1-4 amino acids selected from His, Pro and Gly.

Such multimers may be built from any of the peptides or their variants described herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers (either from the native sequence of human or rabbit HPRG or addition variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced by chemical synthesis, the oligomers preferably have from 2-12 repeats, more preferably 2-8 repeats of the core peptide sequence, and the total number of amino acids in the multimer should not exceed about 110 residues (or their equivalents, when including linkers or spacers).

A preferred synthetic chemical peptide multimer has the formula

P¹

wherein **P¹** is a pentapeptides corresponding to five sequential amino acids from the H/P domain of a mammalian HPRG protein, or substitution or addition variants of these pentapeptides, wherein **n=2-8**, and wherein the pentapeptide alone or in multimeric form has the biological activity of inhibiting cell invasion, endothelial tube formation or angiogenesis in an *in vitro* or *in vivo* bioassay of such activity.

In another embodiment, a preferred synthetic chemical peptide multimer has the formula



P¹ and **P²** are pentapeptides corresponding to five sequential amino acids from the H/P domain of a mammalian HPRG protein, or addition variants of these pentapeptides, wherein (a) **P¹** and **P²** may be the same or different; moreover, each occurrence of **P¹** in the multimer may be different pentapeptides (or variant);

(b) **X** is C₁-C₅ alkyl, C₁-C₅ alkenyl, C₁-C₅ alkynyl, C₁-C₅ polyether containing up to 4 oxygen atoms, wherein **m** = 0 or 1 and **n** = 1-7; **X** may also be Gly_z wherein, **z** = 1-6,

and wherein the pentapeptide alone or in multimeric form has the biological activity of inhibiting cell invasion, endothelial tube formation or angiogenesis in an *in vitro* or *in vivo* bioassay of such activity.

When produced recombinantly, spacers are Gly_z as described above, where z=1-6, and the multimers may have as many repeats of the core peptide sequence as the expression system permits, for example from two to about 100 repeats. A preferred recombinantly produced peptide multimer has the formula:



wherein:

(a) **P¹** and **P²** are pentapeptides corresponding to five sequential amino acids from the H/P domain of a mammalian HPRG protein, or addition variants of these pentapeptides, wherein **P¹** and **P²** may be the same or different; moreover, each occurrence of **P¹** in the multimer may be different pentapeptides (or variant);

wherein $n = 1-100$ and $z = 0-6$;

and wherein the pentapeptide alone or in multimeric form has the biological activity of inhibiting cell invasion, endothelial tube formation or angiogenesis in an *in vitro* or *in vivo* bioassay of such activity.

In the foregoing peptide multimers, **P¹** and **P²** is preferably SEQ ID NO:8, 9 or 10.

The multimer is optionally capped at its N- and C-termini,

It is understood that such multimers may be built from any of the peptides or variants described herein. Although it is preferred that the addition variant monomeric units of the multimer have the biological activity described above, that is not necessary as long as the multimer to which they contribute has the activity.

Diagnostic and Prognostic Compositions

The peptides of the invention can be detectably labeled and used, for example, to detect a peptide binding protein ligand or a cellular binding site/receptor (such as the binding sites on T cells, macrophages or platelets as described above, whether on the surface or in the interior of a cell.

5 The fate of the peptide during and after binding can be followed *in vitro* or *in vivo* by using the appropriate method to detect the label. The labeled peptide may be utilized *in vivo* for diagnosis and prognosis, for example to image occult metastatic foci or for other types of *in situ* evaluations.

The term "diagnostically labeled" means that the polypeptide or peptide has attached to it a diagnostically detectable label. There are many different labels and methods of labeling known to
10 those of ordinary skill in the art, described below. General classes of labels which can be used in the present invention include radioactive isotopes, paramagnetic isotopes, and compounds which can be imaged by positron emission tomography (PET), fluorescent or colored compounds, *etc.* Suitable detectable labels include radioactive, fluorescent, fluorogenic, chromogenic, or other chemical labels. Useful radiolabels (radionuclides), which are detected simply by gamma counter,
15 scintillation counter or autoradiography include ^3H , ^{125}I , ^{131}I , ^{35}S and ^{14}C . ^{131}I is also a useful therapeutic isotope (see below).

A number of U.S. patents, incorporated by reference herein, disclose methods and compositions for complexing metals to larger molecules, including description of useful chelating agents. The metals are preferably detectable metal atoms, including radionuclides, and are
20 complexed to proteins and other molecules. These documents include: US 5,627,286 (Heteroatom-bearing ligands and metal complexes thereof); US 5,618,513 (Method for preparing radiolabeled peptides); US 5,567,408; US 5,443,816 (Peptide-metal ion pharmaceutical preparation and method); US 5,561,220 (Tc- $^{99\text{m}}$ labeled peptides for imaging inflammation).

Common fluorescent labels include fluorescein, rhodamine, dansyl, phycoerythrin,
25 phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine. The fluorophore, such as the dansyl group, must be excited by light of a particular wavelength to fluoresce. See, for example, Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Sixth Ed., Molecular Probes, Eugene, OR., 1996). Fluorescein, fluorescein derivatives and fluorescein-like molecules such as Oregon GreenTM and its derivatives, Rhodamine GreenTM and Rhodol GreenTM, are coupled to
30 amine groups using the isothiocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups. Similarly, fluorophores may also be coupled to thiols using maleimide, iodoacetamide, and

aziridine-reactive groups. The long wavelength rhodamines, which are basically Rhodamine GreenTM derivatives with substituents on the nitrogens, are among the most photostable fluorescent labeling reagents known. Their spectra are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications. This group includes the tetramethylrhodamines, X-rhodamines and Texas RedTM derivatives. Other preferred fluorophores for derivatizing the peptide according to this invention are those which are excited by ultraviolet light. Examples include cascade blue, coumarin derivatives, naphthalenes (of which dansyl chloride is a member), pyrenes and pyridyloxazole derivatives. Also included as labels are two related inorganic materials that have recently been described: semiconductor nanocrystals, comprising, for example, cadmium sulfate (Bruchez, M. *et al.*, *Science* 281:2013-2016 (1998)), and quantum dots, *e.g.*, zinc-sulfide-capped Cd selenide (Chan, W.C.W. *et al.*, *Science* 281:2016-2018 (1998)).

In yet another approach, the amino group of the peptide is allowed to react with reagents that yield fluorescent products, for example, fluorescamine, dialdehydes such as *o*-phthaldialdehyde, naphthalene-2,3-dicarboxylate and anthracene-2,3-dicarboxylate. 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivatives, both chloride and fluoride, are useful to modify amines to yield fluorescent products.

The peptides of the invention can also be labeled for detection using fluorescence-emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the peptide using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA, see Example X, *infra*) or ethylenediaminetetraacetic acid (EDTA). DTPA, for example, is available as the anhydride, which can readily modify the NH_2 -containing peptides of this invention.

For *in vivo* diagnosis or therapy, radionuclides may be bound to the peptide either directly or indirectly using a chelating agent such as DTPA and EDTA. Examples of such radionuclides are ^{99}Tc , ^{123}I , ^{125}I , ^{131}I , ^{111}In , ^{97}Ru , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{72}As , ^{89}Zr , ^{90}Y and ^{201}Tl . Generally, the amount of labeled peptide needed for detectability in diagnostic use will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, contraindications, if any, and other variables, and is to be adjusted by the individual physician or diagnostician. Dosage can vary from 0.01 mg/kg to 100 mg/kg.

The peptide can also be made detectable by coupling to a phosphorescent or a chemiluminescent compound. The presence of the chemiluminescent-tagged peptide is then determined by detecting the presence of luminescence that arises during the course of a chemical

have a binding face (which interacts with any ligand to which HPRG binds) and a non-binding face. Again, similar to HPRG or its peptide, the non-binding face of a peptidomimetic will contain functional groups which can be modified by various therapeutic and diagnostic moieties without modifying the binding face of the peptidomimetic (again, I do not see the description of this for the protein and peptide) ???. A preferred embodiment of a peptidomimetic would contain an aniline on the non-binding face of the molecule. The NH_2 -group of an aniline has a $\text{pK}_a \sim 4.5$ and could therefore be modified by any NH_2 - selective reagent without modifying any NH_2 functional groups on the binding face of the peptidomimetic. Other peptidomimetics may not have any NH_2 functional groups on their binding face and therefore, any NH_2 , without regard for pK_a could be displayed on the non-binding face as a site for conjugation. In addition other modifiable functional groups, such as $-\text{SH}$ and $-\text{COOH}$ could be incorporated into the non-binding face of a peptidomimetic as a site of conjugation. A therapeutic or diagnostic moiety could also be directly incorporated during the synthesis of a peptidomimetic and preferentially be displayed on the non-binding face of the molecule.

This invention also includes compounds that retain partial peptide characteristics. For example, any proteolytically unstable bond within a peptide of the invention could be selectively replaced by a non-peptidic element such as an isostere (N-methylation; D-amino acid) or a reduced peptide bond while the rest of the molecule retains its peptide nature.

Peptidomimetic compounds, either agonists, substrates or inhibitors, have been described for a number of bioactive peptides such as opioid peptides, VIP, thrombin, HIV protease, *etc.* Methods for designing and preparing peptidomimetic compounds are known in the art (Hruby, V.J., *Biopolymers* 33:1073-1082 (1993); Wiley, R.A. *et al.*, *Med. Res. Rev.* 13:327-384 (1993); Moore *et al.*, *Adv. in Pharmacol* 33:91-141 (1995); Giannis *et al.*, *Adv. in Drug Res.* 29:1-78 (1997), which references are incorporated by reference in their entirety). These methods are used to make peptidomimetics that possess at least the binding capacity and specificity of the HPRG peptides and preferably also possess the biological activity. Knowledge of peptide chemistry and general organic chemistry available to those skilled in the art are sufficient, in view of the present disclosure, for designing and synthesizing such compounds.

For example, such peptidomimetics may be identified by inspection of the crystallographically-derived three-dimensional structure of a peptide of the invention either free or bound in complex with a ligand such as (a) heparin, plasminogen, fibrinogen, vitronectin and

thrombospondin or (b) small ligands, such as heme and transition metal ions (zinc, copper and nickel). Alternatively, the structure of a peptide of the invention bound to its ligand can be gained by the techniques of nuclear magnetic resonance spectroscopy. The better knowledge of the stereochemistry of the interaction of the peptide with its ligand or receptor will permit the rational design of such peptidomimetic agents. The structure of a peptide or protein of the invention in the absence of ligand could also provide a scaffold for the design of mimetic molecules.

ANTIBODIES SPECIFIC FOR EPITOPES OF HPRG

The present invention provides antibodies, both polyclonal and monoclonal, reactive with an epitope of HPRG, preferably, an epitope of the H/P domain. The antibodies, referred to herein as “anti-H/P antibodies” may be xenogeneic, allogeneic, syngeneic, or modified forms thereof, such as humanized or chimeric antibodies. Antiidiotypic antibodies specific for the idiotype of an anti-HPRG antibody are also included.

In the following description, reference will be made to various methodologies known to those of skill in the art of immunology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full. Standard reference works setting forth the general principles of immunology include A.K. Abbas *et al.*, *Cellular and Molecular Immunology* (Fourth Ed.), W.B. Saunders Co., Philadelphia, 2000; C.A. Janeway *et al.*, *Immunobiology. The Immune System in Health and Disease*, Fourth ed., Garland Publishing Co., New York, 1999; Roitt, I. *et al.*, *Immunology*, (current ed.) C.V. Mosby Co., St. Louis, MO (1999); Klein, J., *Immunology*, Blackwell Scientific Publications, Inc., Cambridge, MA, (1990).

Monoclonal antibodies (mAbs) and methods for their production and use are described in Kohler and Milstein, *Nature* 256:495-497 (1975); U.S. Patent No. 4,376,110; Hartlow, E. *et al.*, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988); *Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, New York, NY (1980); H. Zola *et al.*, in *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, 1982)).

Anti-idiotypic antibodies are described, for example, in *Idiotypy in Biology and Medicine*, Academic Press, New York, 1984; *Immunological Reviews* Volume 79, 1984; *Immunological Reviews* Volume 90, 1986; *Curr. Top. Microbiol., Immunol.* Volume 119, 1985; Bona, C. *et al.*, *CRC Crit. Rev. Immunol.*, pp. 33-81 (1981); Jerne, NK, *Ann. Immunol.* 125C:373-389 (1974); Jerne,

NK, In: *Idiotypes - Antigens on the Inside*, Westen-Schnurr, I., ed., Editiones Roche, Basel, 1982, Urbain, J *et al.*, *Ann. Immunol.* 133D:179- (1982); Rajewsky, K. *et al.*, *Ann. Rev. Immunol.* 1:569-607 (1983)

The term “antibody” is also meant to include both intact molecules as well as fragments thereof that include the antigen-binding site and are capable of binding to a HPRG epitope. These include , Fab and F(ab')₂ fragments which lack the Fc fragment of an intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)). Also included are Fv fragments (Hochman, J. *et al.* (1973) *Biochemistry* 12:1130-1135; Sharon, J. *et al.* (1976) *Biochemistry* 15:1591-1594).). These various fragments are to be produced using conventional techniques such as protease cleavage or chemical cleavage (see, *e.g.*, Rousseaux *et al.*, *Meth. Enzymol.*, 121:663-69 (1986))

Polyclonal antibodies are obtained as sera from immunized animals such as rabbits, goats, rodents, *etc.* and may be used directly without further treatment or may be subjected to conventional enrichment or purification methods such as ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see Zola *et al.*, *supra*).

The immunogen used to produce the present anti-H/P antibodies may comprise the complete HPRG protein, or fragments or derivatives thereof. Preferred immunogens comprise all or a part of the H/P central domain of HPRG. Immunogens comprising this domain are produced in a variety of ways known in the art, *e.g.*, expression of cloned genes using conventional recombinant methods, isolation from cells of origin, cell populations expressing high levels of HPRG, *etc.*

The mAbs may be produced using conventional hybridoma technology, such as the procedures introduced by Kohler and Milstein (*supra*) and modifications thereof (see above references). An animal, preferably a mouse is primed by immunization with an immunogen as above to elicit the desired antibody response in the primed animal.

25 B lymphocytes from the lymph nodes, spleens or peripheral blood of a primed, animal are fused with myeloma cells, generally in the presence of a fusion promoting agent such as polyethylene glycol (PEG). Any of a number of murine myeloma cell lines are available for such use: the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines (available from the ATCC, Rockville, MD). Subsequent steps include growth in selective medium so that unfused parental myeloma cells and donor lymphocyte cells eventually die while only the hybridoma cells survive. These are cloned and grown and their supernatants screened for the

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presence of antibody of the desired specificity, *e.g.*, by immunoassay techniques using the HPRG protein. Positive clones are subcloned, *e.g.*, by limiting dilution, and the mAbs are isolated.

Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art (see generally Fink *et al.*, *Prog. Clin. Pathol.*, 9:121-33 (1984)). Generally, the individual cell line is propagated in culture and the culture medium containing high concentrations of a single mAb can be harvested by decantation, filtration, or centrifugation.

The antibody may be produced as a single chain antibody or scFv instead of the normal multimeric structure. Single chain antibodies include the hypervariable regions from an Ig of interest and recreate the antigen binding site of the native Ig while being a fraction of the size of the intact Ig (Skerra, A. *et al.* (1988) *Science*, 240: 1038-1041; Pluckthun, A. *et al.* (1989) *Methods Enzymol.* 178: 497-515; Winter, G. *et al.* (1991) *Nature*, 349: 293-299); Bird *et al.*, (1988) *Science* 242:423; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879; Jost CR *et al.*, *J Biol Chem.* 1994 269:26267-26273; U.S. Patents No. 4,704,692, 4,853,871, 4,94,6778, 5,260,203, 5,455,030). DNA sequences encoding the V regions of the H chain and the L chain are ligated to a linker encoding at least about 4 amino acids (typically small neutral amino acids). The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody.

For *in vivo* use, particularly for injection into humans, it is desirable to decrease the immunogenicity of the mAb by humanizing the antibodies using methods known in the art. The humanized antibody may be the product of an animal having transgenic human Ig Constant region genes (see for example WO 90/10077 and WO 90/04036). Alternatively, the antibody of interest may be genetically engineered to substitute the CH₁, CH₂, CH₃, hinge domains, and/or the framework domain with the corresponding human sequence (see WO 92/02190).

Antibodies can be selected for particular desired properties. In the case of an antibody to be used for therapy, antibody screening procedures can include any of the *in vitro* or *in vivo* bioassays that measure angiogenesis, cell invasion, and the like. Moreover, the antibodies may be screened in various of the tumor models described herein to see if they promote or inhibit angiogenesis (or resultant tumor growth or metastasis). In this way, antibodies that are HPRG mimics or antagonists can be selected. Thus, the present invention includes therapeutic antibodies (discussed in more

detail below) that promote angiogenesis by binding to and otherwise inhibiting the action of HPRG or its H/P domain.

Use of Antibodies to Detect Free H/P Domain of HPRG

Antibodies specific for an epitope of the H/P domain are useful in immunoassays to detect molecules containing these epitopes in a body fluid or sample, preferably serum or plasma. Such antibodies would detect HPRG, a cleaved H/P domain of HPRG or an epitope-bearing fragment of the domain. Thus, if proteolysis in the tumor milieu results in release of the H/P domain plasma (just in case proteolysis releases free H/P in the tumor milieu) or in tissue.

By measuring the levels of H/P domain released from HPRG, the antibodies and immunoassays of this invention are used diagnostically to monitor the progress of a disease, where H/P domain levels may reflect the amount of tumor tissue present.

Any conventional immunoassay known in the art may be employed for this purpose, though Enzyme Immunoassays such as ELISA are preferred. Immunoassay methods are also described in Coligan, J.E. *et al.*, eds., *Current Protocols in Immunology*, Wiley-Interscience, New York 1991 (or current edition); Butt, W.R. (ed.) *Practical Immunoassay: The State of the Art*, Dekker, New York, 1984; Bizollon, Ch. A., ed., *Monoclonal Antibodies and New Trends in Immunoassays*, Elsevier, New York, 1984; Butler, J.E., ELISA (Chapter 29), In: van Oss, C.J. *et al.*, (eds), *IMMUNOCHEMISTRY*, Marcel Dekker, Inc., New York, 1994, pp. 759-803; Butler, J.E. (ed.), *Immunochemistry of Solid-Phase Immunoassay*, CRC Press, Boca Raton, 1991; Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986; Work, T.S. *et al.*, *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, NY, (1978) (Chapter by Chard, T., "An Introduction to Radioimmune Assay and Related Techniques").

In Vitro Testing of Compositions

A. Assay for endothelial cell migration

For EC migration, transwells are coated with type I collagen (50 $\mu\text{g}/\text{mL}$) by adding 200 μL of the collagen solution per transwell, then incubating overnight at 37°C. The transwells are assembled in a 24-well plate and a chemoattractant (*e.g.*, FGF-2) is added to the bottom chamber in a total volume of 0.8 mL media. ECs, such as human umbilical vein endothelial cells (HUVEC), which have been detached from monolayer culture using trypsin, are diluted to a final concentration of about 10^6 cells/mL with serum-free media and 0.2 mL of this cell suspension is added to the

upper chamber of each transwell. Inhibitors to be tested are added to both the upper and lower chambers, and the migration is allowed to proceed for 5 hrs in a humidified atmosphere at 37°C. The transwells are removed from the plate stained using DiffQuik®. Cells which did not migrate are removed from the upper chamber by scraping with a cotton swab and the membranes are detached, mounted on slides, and counted under a high-power field (400x) to determine the number of cells migrated.

B. Biological Assay of Anti-Invasive Activity

The compositions of the invention are tested for their anti-invasive capacity. The ability of cells such as ECs or tumor cells (*e.g.*, PC-3 human prostatic carcinoma) cells to invade through a reconstituted basement membrane (Matrigel®) in an assay known as a Matrigel® invasion assay system as described in detail by Kleinman *et al.*, *Biochemistry* 25: 312-318, 1986 and Parish *et al.*, *Int. J. Cancer* 52:378-383, 1992. Matrigel® is a reconstituted basement membrane containing type IV collagen, laminin, heparan sulfate proteoglycans such as perlecan, which bind to and localize bFGF, vitronectin as well as transforming growth factor-β (TGFβ), urokinase-type plasminogen activator (uPA), tissue plasminogen activator (tPA), and the serpin known as plasminogen activator inhibitor type 1 (PAI-1) (Chambers *et al.*, *Canc. Res.* 55:1578-1585, 1995). It is accepted in the art that results obtained in this assay for compounds which target extracellular receptors or enzymes are predictive of the efficacy of these compounds *in vivo* (Rabbani *et al.*, *Int. J. Cancer* 63: 840-845, 1995).

Such assays employ transwell tissue culture inserts. Invasive cells are defined as cells which are able to traverse through the Matrigel® and upper aspect of a polycarbonate membrane and adhere to the bottom of the membrane. Transwells (Costar) containing polycarbonate membranes (8.0 μm pore size) are coated with Matrigel® (Collaborative Research), which has been diluted in sterile PBS to a final concentration of 75 μg/mL (60 μL of diluted Matrigel® per insert), and placed in the wells of a 24-well plate. The membranes are dried overnight in a biological safety cabinet, then rehydrated by adding 100 μL of DMEM containing antibiotics for 1 hour on a shaker table. The DMEM is removed from each insert by aspiration and 0.8 mL of DMEM/10 % FBS/antibiotics is added to each well of the 24-well plate such that it surrounds the outside of the transwell ("lower chamber"). Fresh DMEM/ antibiotics (100μL), human Glu-plasminogen (5 μg/mL), and any inhibitors to be tested are added to the top, inside of the transwell ("upper chamber"). The cells which are to be tested are trypsinized and resuspended in DMEM/antibiotics, then added to the top

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C. Tube-Formation Assays of Anti-Angiogenic Activity

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Caspase-3 activity

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***In Vivo* Study of the HPRG Peptides**

A. Corneal Angiogenesis Model

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incisions made in the cornea 1.0-1.5 mm from the limbus. Neovascularization is assessed at 5 and 7 days after implantation. On day 7, animals are anesthetized and infused with a dye such as colloidal carbon to stain the vessels. The animals are then euthanized, the corneas fixed with formalin, and the corneas flattened and photographed to assess the degree of neovascularization. Neovessels may be quantitated by imaging the total vessel area or length or simply by counting vessels.

B. Matrigel® Plug Assay

This assay is performed essentially as described by Passaniti *et al.* (*Lab Invest.* 67:519-528 (1992)). Ice-cold Matrigel® (*e.g.*, 500 µL) (Collaborative Biomedical Products, Inc., Bedford, MA) is mixed with heparin (*e.g.*, 50 µg/ml), FGF-2 (*e.g.*, 400 ng/ml) and the compound to be tested. In some assays, bFGF may be substituted with tumor cells as the angiogenic stimulus. The Matrigel® mixture is injected subcutaneously into 4-8 week-old athymic nude mice at sites near the abdominal midline, preferably 3 injections per mouse. The injected Matrigel® forms a palpable solid gel. Injection sites are chosen such that each animal receives a positive control plug (such as FGF-2 + heparin), a negative control plug (*e.g.*, buffer + heparin) and a plug that includes the compound being tested for its effect on angiogenesis, *e.g.*, (FGF-2 + heparin + compound). All treatments are preferably run in triplicate. Animals are sacrificed by cervical dislocation at about 7 days post injection or another time that may be optimal for observing angiogenesis. The mouse skin is detached along the abdominal midline, and the Matrigel® plugs are recovered and scanned immediately at high resolution. Plugs are then dispersed in water and incubated at 37°C overnight. Hemoglobin (Hb) levels are determined using Drabkin's solution (*e.g.*, obtained from Sigma) according to the manufacturers' instructions. The amount of Hb in the plug is an indirect measure of angiogenesis as it reflects the amount of blood in the sample. In addition, or alternatively, animals may be injected prior to sacrifice with a 0.1 ml buffer (preferably PBS) containing a high molecular weight dextran to which is conjugated a fluorophore. The amount of fluorescence in the dispersed plug, determined fluorimetrically, also serves as a measure of angiogenesis in the plug. Staining with mAb anti-CD31 (CD31 is "platelet-endothelial cell adhesion molecule or PECAM") may also be used to confirm neovessel formation and microvessel density in the plugs.

C. Chick chorioallantoic membrane (CAM) angiogenesis assay

This assay is performed essentially as described by Nguyen *et al.* (*Microvascular Res.* 47:31-40 (1994)). A mesh containing either angiogenic factors (bFGF) or tumor cells plus inhibitors is placed onto the CAM of an 8-day old chick embryo and the CAM observed for 3-9

days after implantation of the sample. Angiogenesis is quantitated by determining the percentage of squares in the mesh which contain blood vessels.

D. In Vivo Assessment Angiogenesis Inhibition and Anti-Tumor Effects Using the Matrigel® Plug Assay with Tumor Cells

5 In this assay, tumor cells, for example $1-5 \times 10^6$ cells of the 3LL Lewis lung carcinoma or the rat prostate cell line MatLyLu, are mixed with Matrigel® and then injected into the flank of a mouse following the protocol described in Sec. B., above. A mass of tumor cells and a powerful angiogenic response can be observed in the plugs after about 5 to 7 days. The anti-tumor and anti-angiogenic action of a compound in an actual tumor environment can be evaluated by including it in
10 the plug. Measurement is then made of tumor weight, Hb levels or fluorescence levels (of a dextran-fluorophore conjugate injected prior to sacrifice). To measure Hb or fluorescence, the plugs are first homogenize with a tissue homogenizer.

E. Xenograft model of subcutaneous (s.c.) tumor growth

Nude mice are inoculated with MDA-MB-231 cells (human breast carcinoma) and Matrigel® (1 x 10⁶ cells in 0.2mL) s.c. in the right flank of the animals. The tumors are staged to 200 mm³ and then treatment with a test composition is initiated (100µg/animal/day given q.d. IP). Tumor volumes are obtained every other day and the animals are sacrificed after 2 weeks of treatment. The tumors are excised, weighed and paraffin embedded. Histological sections of the tumors are analyzed by H and E, anti-CD31, Ki-67, TUNEL, and CD68 staining.

20 F. Xenograft Model of Metastasis

The compounds of this invention are also tested for inhibition of late metastasis using an experimental metastasis model (Crowley, C.W. *et al.*, *Proc. Natl. Acad. Sci. USA* 90 5021-5025 (1993)). Late metastasis involves the steps of attachment and extravasation of tumor cells, local invasion, seeding, proliferation and angiogenesis. Human prostatic carcinoma cells (PC-3) transfected with a reporter gene, preferably the green fluorescent protein (GFP) gene, but as an alternative with a gene encoding the enzymes chloramphenicol acetyl-transferase (CAT), luciferase or LacZ, are inoculated into nude mice. This approach permits utilization of either of these markers (fluorescence detection of GFP or histochemical colorimetric detection of enzymatic activity) to follow the fate of these cells. Cells are injected, preferably iv, and metastases identified after about 14 days, particularly in the lungs but also in regional lymph nodes, femurs and brain. This mimics the organ tropism of naturally occurring metastases in human prostate cancer. For example, GFP-

expressing PC-3 cells (1×10^6 cells per mouse) are injected iv into the tail veins of nude (*nu/nu*) mice. Animals are treated with a test composition at $100\mu\text{g}/\text{animal}/\text{day}$ given q.d. IP. Single metastatic cells and foci are visualized and quantitated by fluorescence microscopy or light microscopic histochemistry or by grinding the tissue and quantitative colorimetric assay of the detectable label.

G. Inhibition of Spontaneous Metastasis *In Vivo* by HPRG and Functional Derivatives
 The rat syngeneic breast cancer system (Xing *et al.*, *Int. J. Cancer* 67:423-429 (1996))

employs Mat BIII rat breast cancer cells. Tumor cells, for example about 10^6 suspended in 0.1 mL PBS, are inoculated into the mammary fat pads of female Fisher rats. At the time of inoculation, a 14-day Alza osmotic mini-pump is implanted intraperitoneally to dispense the test compound. The compound is dissolved in PBS (*e.g.*, 200 mM stock), sterile filtered and placed in the minipump to achieve a release rate of about 4 mg/kg/day. Control animals receive vehicle (PBS) alone or a vehicle control peptide in the minipump. Animals are sacrificed at about day 14.

Therapeutic outcomes

In the rats treated with the active compounds of the present invention, significant reductions in the size of the primary tumor and in the number of metastases in the spleen, lungs, liver, kidney and lymph nodes (enumerated as discrete foci) are observed. Histological and immunohistochemical analysis reveal increased necrosis and signs of apoptosis in tumors in treated animals. Large necrotic areas are seen in tumor regions lacking neovascularization. Human or rabbit HPRG and their derivatives to which ^{131}I is conjugated (either 1 or 2 I atoms per molecule of peptide) are effective radiotherapeutics and are found to be at least two-fold more potent than the unconjugated polypeptides. In contrast, treatment with control peptides fails to cause a significant change in tumor size or metastasis.

H. 3LL Lewis Lung Carcinoma: Primary Tumor Growth

This tumor line arose spontaneously in 1951 as carcinoma of the lung in a C57BL/6 mouse (*Cancer Res* 15:39, 1955. See, also Malave, I. *et al.*, *J. Nat'l. Canc. Inst.* 62:83-88 (1979)). It is propagated by passage in C57BL/6 mice by subcutaneous (sc) inoculation and is tested in semiallogeneic C57BL/6 x DBA/2 F₁ mice or in allogeneic C3H mice. Typically six animals per group for subcutaneously (sc) implant, or ten for intramuscular (im) implant are used. Tumor may be implanted sc as a 2-4 mm fragment, or im or sc as an inoculum of suspended cells of about $0.5\text{-}2 \times 10^6$ -cells. Treatment begins 24 hours after implant or is delayed until a tumor of specified size

(usually approximately 400 mg) can be palpated. The test compound is administered ip daily for 11 days

Animals are followed by weighing, palpation, and measurement of tumor size. Typical tumor weight in untreated control recipients on day 12 after im inoculation is 500-2500 mg.

Typical median survival time is 18-28 days. A positive control compound, for example cyclophosphamide at 20 mg/kg/injection per day on days 1-11 is used. Results computed include mean animal weight, tumor size, tumor weight, survival time. For confirmed therapeutic activity, the test composition should be tested in two multi-dose assays.

I. 3LL Lewis Lung Carcinoma: Primary Growth and Metastasis Model

This model has been utilized by a number of investigators. See, for example, Gorelik, E. *et al.*, *J. Nat'l. Canc. Inst.* 65:1257-1264 (1980); Gorelik, E. *et al.*, *Rec. Results Canc. Res.* 75:20-28 (1980); Isakov, N. *et al.*, *Invasion Metas.* 2:12-32 (1982); Talmadge J.E. *et al.*, *J. Nat'l. Canc. Inst.* 69:975-980 (1982); Hilgard, P. *et al.*, *Br. J. Cancer* 35:78-86(1977)). Test mice are male C57BL/6 mice, 2-3 months old. Following sc, im, or intra-footpad implantation, this tumor produces metastases, preferentially in the lungs. With some lines of the tumor, the primary tumor exerts anti-metastatic effects and must first be excised before study of the metastatic phase (see also U.S. 5,639,725).

Single-cell suspensions are prepared from solid tumors by treating minced tumor tissue with a solution of 0.3% trypsin. Cells are washed 3 times with PBS (pH 7.4) and suspended in PBS.

Viability of the 3LL cells prepared in this way is generally about 95-99% (by trypan blue dye exclusion). Viable tumor cells (3×10^4 - 5×10^6) suspended in 0.05 ml PBS are injected subcutaneously, either in the dorsal region or into one hind foot pad of C57BL/6 mice. Visible tumors appear after 3-4 days after dorsal sc injection of 10^6 cells. The day of tumor appearance and the diameters of established tumors are measured by caliper every two days.

The treatment is given as one or two doses of peptide or derivative, per week. In another embodiment, the peptide is delivered by osmotic minipump.

In experiments involving tumor excision of dorsal tumors, when tumors reach about 1500 mm³ in size, mice are randomized into two groups: (1) primary tumor is completely excised; or (2) sham surgery is performed and the tumor is left intact. Although tumors from 500-3000 mm³ inhibit growth of metastases, 1500 mm³ is the largest size primary tumor that can be safely resected with high survival and without local regrowth. After 21 days, all mice are sacrificed and autopsied.

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had been repeatedly observed (for example, see U.S. 5,639,725). These observations have implications for the prognosis of patients who undergo cancer surgery.

For a compound to be useful in accordance with this invention, it should demonstrate activity in at least one of the above (*in vitro* or *in vivo*) assay systems.

5 Pharmaceutical and Therapeutic Compositions and Their Administration

The compounds that may be employed in the pharmaceutical compositions of the invention include all of the polypeptide and peptide compounds described above, as well as the pharmaceutically acceptable salts of these compounds. Pharmaceutically acceptable acid addition salts of the compounds of the invention containing a basic group are formed where appropriate with strong or moderately strong, non-toxic, organic or inorganic acids by methods known to the art. Exemplary of the acid addition salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate and nitrate salts.

Pharmaceutically acceptable base addition salts of compounds of the invention containing an acidic group are prepared by known methods from organic and inorganic bases and include, for example, nontoxic alkali metal and alkaline earth bases, such as calcium, sodium, potassium and ammonium hydroxide; and nontoxic organic bases such as triethylamine, butylamine, piperazine, and tri(hydroxymethyl)methylamine.

As stated above, the compounds of the invention possess the ability to inhibit endothelial cell proliferation, motility, or invasiveness and angiogenesis, properties that are exploited in the treatment of cancer, in particular metastatic cancer. A composition of this invention may be active *per se*, or may act as a “pro-drug” that is converted *in vivo* to the active form.

Therapeutically Labeled Compositions

In a preferred embodiment, the polypeptide and peptides describe herein are “therapeutically conjugated” or “therapeutically labeled” (terms which are intended to be interchangeable) and used to deliver a therapeutic agent to the site to which the compounds home and bind, such as sites of tumor metastasis or foci of infection/inflammation, restenosis or fibrosis. The term “therapeutically conjugated” means that the modified peptide is conjugated to another therapeutic agent that is directed either to the underlying cause or to a “component” of tumor invasion, angiogenesis, inflammation or other pathology. A therapeutically labeled protein or peptide carries a suitable therapeutic “label” also referred to herein as a “therapeutic moiety.” A therapeutic

moiety is an atom, a molecule, a compound or any chemical component added to the peptide that renders it active in treating a target disease or condition, primarily one associated with undesired angiogenesis. As noted above, the peptides of the present invention are prepared by conventional means, either chemical synthesis, proteolysis of HPRG or recombinant means. The therapeutic moiety may be bound directly or indirectly to the peptide. The therapeutically labeled protein or peptide is administered as pharmaceutical composition which comprises a pharmaceutically acceptable carrier or excipient, and is preferably in a form suitable for injection.

Examples of useful therapeutic radioisotopes (ordered by atomic number) include ^{47}Sc , ^{67}Cu , ^{90}Y , ^{109}Pd , ^{125}I , ^{131}I , ^{186}Re , ^{188}Re , ^{199}Au , ^{211}At , ^{212}Pb and ^{217}Bi . These atoms can be conjugated to the peptide directly, indirectly as part of a chelate, or, in the case of iodine, indirectly as part of an iodinated Bolton-Hunter group. The radioiodine can be introduced either before or after this group is coupled to the peptide compound.

Preferred doses of the radionuclide conjugates are a function of the specific radioactivity to be delivered to the target site which varies with tumor type, tumor location and vascularization, kinetics and biodistribution of the peptide carrier, energy of radioactive emission by the nuclide, *etc.* Those skilled in the art of radiotherapy can readily adjust the dose of the peptide in conjunction with the dose of the particular nuclide to effect the desired therapeutic benefit without undue experimentation.

Another therapeutic approach included here is the use of boron neutron capture therapy, where a boronated peptide is delivered to a desired target site, such as a tumor, most preferably an intracranial tumor (Barth, R.F., *Cancer Invest.* 14:534-550 (1996); Mishima, Y. (ed.), *Cancer Neutron Capture Therapy*, New York: Plenum Publishing Corp., 1996; Soloway, A.H., *et al.*, (eds), *J. Neuro-Oncol.* 33:1-188 (1997). The stable isotope ^{10}B is irradiated with low energy ($<0.025\text{eV}$) thermal neutrons, and the resulting nuclear capture yields α -particles and ^7Li nuclei which have high linear energy transfer and respective path lengths of about 9 and 5 μm . This method is predicated on ^{10}B accumulation in the tumor with lower levels in blood, endothelial cells and normal tissue (*e.g.*, brain). Such delivery has been accomplished using epidermal growth factor (Yang, W. *et al.*, *Cancer Res* 57:4333-4339 (1997).

Other therapeutic agents which can be coupled to the peptide compounds according to the method of the invention are drugs, prodrugs, enzymes for activating pro-drugs, photosensitizing agents, nucleic acid therapeutics, antisense vectors, viral vectors, lectins and other toxins.

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may be incorporated into convenient dosage forms, such as capsules, impregnated wafers, tablets or

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5 may be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company,
Easton Pennsylvania (Gennaro 18th ed. 1990).

the desired products for oral, parenteral, topical, transdermal, intravaginal, intrapenile, intranasal, intrabronchial, intracranial, intraocular, intraaural and rectal administration. The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and so forth.

15 animal genera and species, and are equally applicable in the practice of human or veterinary medicine. Thus, the pharmaceutical compositions can be used to treat domestic and commercial animals, including birds and more preferably mammals, as well as humans.

introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body, such as intravenous (i.v.) injection or infusion. "Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ. Examples include intravaginal, intrapenile, intranasal, intrabronchial (or lung instillation), intracranial, intra-aural or intraocular.

The term "local administration" refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous (s.c.) injections, intramuscular (i.m.) injections. One of skill in the art would understand that local administration or regional administration often also result in entry of a composition into the circulatory system, *i.e.*, so that s.c. or i.m. are also routes for systemic administration. Injectables or infusible preparations can be prepared in conventional forms, either as solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection or infusion, or as

emulsions. Though the preferred routes of administration are systemic, such as i.v., the pharmaceutical composition may be administered topically or transdermally, *e.g.*, as an ointment, cream or gel; orally; rectally; *e.g.*, as a suppository.

For topical application, the compound may be incorporated into topically applied vehicles such as a salve or ointment. The carrier for the active ingredient may be either in sprayable or nonsprayable form. Non-sprayable forms can be semi-solid or solid forms comprising a carrier indigenous to topical application and having a dynamic viscosity preferably greater than that of water. Suitable formulations include, but are not limited to, solution, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like. If desired, these may be sterilized or mixed with auxiliary agents, *e.g.*, preservatives, stabilizers, wetting agents, buffers, or salts for influencing osmotic pressure and the like. Preferred vehicles for non-sprayable topical preparations include ointment bases, *e.g.*, polyethylene glycol-1000 (PEG-1000); conventional creams such as HEB cream; gels; as well as petroleum jelly and the like.

Also suitable for topic application as well as for lung instillation are sprayable aerosol preparations wherein the compound, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, perfumes, and/or antioxidants in addition to the compounds of the invention.

For the preferred topical applications, especially for humans, it is preferred to administer an effective amount of the compound to an affected area, *e.g.*, skin surface, mucous membrane, eyes, *etc.* This amount will generally range from about 0.001 mg to about 1 g per application, depending upon the area to be treated, the severity of the symptoms, and the nature of the topical vehicle employed.

Other pharmaceutically acceptable carriers for polypeptide or nucleic acid compositions of the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active polypeptide or peptide, or the nucleic acid is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate,

stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

Therapeutic compositions for treating tumors and cancer may comprise, in addition to the peptide, one or more additional anti-tumor agents, such as mitotic inhibitors, *e.g.*, vinblastine; alkylating agents, *e.g.*, cyclophosphamide; folate inhibitors, *e.g.*, methotrexate, piritrexim or trimetrexate; antimetabolites, *e.g.*, 5-fluorouracil and cytosine arabinoside; intercalating antibiotics, *e.g.*, adriamycin and bleomycin; enzymes or enzyme inhibitors, *e.g.*, asparaginase, topoisomerase inhibitors such as etoposide; or biological response modifiers, *e.g.*, interferons or interleukins. In fact, pharmaceutical compositions comprising any known cancer therapeutic in combination with the peptides disclosed herein are within the scope of this invention. The pharmaceutical composition may also comprise one or more other medicaments to treat additional symptoms for which the target patients are at risk, for example, anti-infectives including antibacterial, anti-fungal, anti-parasitic, anti-viral, and anti-coccidial agents.

The therapeutic dosage administered is an amount which is therapeutically effective, as is known to or readily ascertainable by those skilled in the art. The dose is also dependent upon the age, health, and weight of the recipient, kind of concurrent treatment(s), if any, the frequency of treatment, and the nature of the effect desired, such as, for example, anti-inflammatory effects or anti-bacterial effect.

As discussed above, antibodies specific for epitopes of the H/P domain, by inhibiting the anti-angiogenic effects of HPRG via the H/P domain, are useful in the induction of neovascularization and can be used to treat diseases or conditions in which increased angiogenesis is desired. Such conditions include coronary artery disease and peripheral artery disease, in which therapeutic angiogenesis is known to be beneficial (Freedman SB and Isner JM, *Ann Intern Med*, 2002, 136:54-71 and *J Mol Cell Cardiol*, 2001 33:379-393; Durairaj, A. *et al.*, *Cardiol Rev*, 2000, 8:279-287; Emanueli C *et al.*, *Br J Pharmacol*, 2001, 133:951-958; Isner, JM *et al.*, *Hum Gene Ther*, 1996, 7:959-88). In general, any form of tissue ischemia resulting from vascular occlusion, vascular disease or surgery can be treated in this manner (Isner *et al.*, *supra*; Webster KA., *Crit Rev Eukaryot Gene Expr*, 2000, 10:113-125), for example peripheral limb ischemia or hepatic arterial occlusion in liver transplantation (Yedlicka, JW *et al.*, *J Vasc Interv Radiol*, 1991, 2:235-240) where the present antibodies will promote revascularization of ischemic tissues.

These antibodies are useful in the promotion of wound healing (including recovery from surgical wounds), which is known to be dependent upon angiogenic processes (Liekens S *et al.*, *Biochem Pharmacol*, 2001, 61:253-270; Lingen, MW, *Arch Pathol Lab Med*, 2001, 125:67-71; Raza SL *et al.*, *J Investig Dermatol Symp Proc*, 2000, 5:47-54; Tonnesen MG *et al.*, *J Investig Dermatol Symp Proc*, 2000, 5:40-46; Hunt TK, *Adv Skin Wound Care*, 2000, 13(2 Suppl):6-11; Grant DS *et al.*, *Adv Exp Med Biol*, 2000, 476:139-154; Drixler TA *et al.*, *Eur J Surg*, 2000, 166:435-446; Singer AJ *et al.*, *N Engl J Med*, 1999, 341:738-746; Martin, P, *Science*, 1997, 276:75-81) and in accelerating or enhancing fracture repair (Glowacki, J, *Clin Orthop*, 1998, 355 Suppl:S82-89).

Anti-H/P antibodies can be used in conjunction with cellular therapy and transplantation of pancreatic islet cells in the treatment of diabetes as vascular endothelium acts to stimulate or induce pancreatic organogenesis and insulin production by pancreatic beta cells (Lammert E *et al.*, *Science*, 2001, 294:564-567; see also page 530-531). Liver organogenesis is also promoted by vasculogenic endothelial cells and nascent vessels (Matsumoto, K. *et al.*, *Science*, 2001, 294:559-563). See also, DeFrancesco, L., *The Scientist* 15:17 (2001).

Screening of antibodies or supernatants of hybridoma cultures to detect anti-H/P antibodies with the desired pro-angiogenic activity are performed using the *in vitro* and *in vivo* bioassays described above, such as the Matrigel® plug assay.

Therapeutic Methods

The methods of this invention may be used to inhibit tumor growth and invasion in a subject or to suppress angiogenesis induced by tumors by inhibiting endothelial cell growth and migration. By inhibiting the growth or invasion of a tumor or angiogenesis, the methods result in inhibition of tumor metastasis. A vertebrate subject, preferably a mammal, more preferably a human, is administered an amount of the compound effective to inhibit tumor growth, invasion or angiogenesis. The compound or pharmaceutically acceptable salt thereof is preferably administered in the form of a pharmaceutical composition as described above.

Doses of the proteins (including antibodies), peptides, peptide multimers, *etc.*, preferably include pharmaceutical dosage units comprising an effective amount of the peptide. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material (*e.g.*, the HPRG-derived domain or peptide, or nucleic acid encoding the polypeptide) calculated to produce the desired therapeutic effect, in

association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

By an effective amount is meant an amount sufficient to achieve a steady state concentration *in vivo* which results in a measurable reduction in any relevant parameter of disease and may include growth of primary or metastatic tumor, any accepted index of inflammatory reactivity, or a measurable prolongation of disease-free interval or of survival. For example, a reduction in tumor growth in 20 % of patients is considered efficacious (Frei III, E., *The Cancer Journal* 3:127-136 (1997)). However, an effect of this magnitude is not considered to be a minimal requirement for the dose to be effective in accordance with this invention.

In one embodiment, an effective dose is preferably 10-fold and more preferably 100-fold higher than the 50% effective dose (ED₅₀) of the compound in an *in vivo* assay as described herein.

The amount of active compound to be administered depends on the precise peptide or derivative selected, the disease or condition, the route of administration, the health and weight of the recipient, the existence of other concurrent treatment, if any, the frequency of treatment, the nature of the effect desired, for example, inhibition of tumor metastasis, and the judgment of the skilled practitioner.

A preferred dose for treating a subject, preferably mammalian, more preferably human, with a tumor is an amount of up to about 100 milligrams of active protein or peptide-based compound per kilogram of body weight. A typical single dosage of the peptide or peptidomimetic is between about 1 ng and about 100mg/kg body weight. For topical administration, dosages in the range of about 0.01-20% concentration (by weight) of the compound, preferably 1-5%, are suggested. A total daily dosage in the range of about 0.1 milligrams to about 7 grams is preferred for intravenous administration. The foregoing ranges are, however, suggestive, as the number of variables in an individual treatment regime is large, and considerable excursions from these preferred values are expected.

An effective amount or dose of the peptide for inhibiting endothelial cell proliferation or migration *in vitro* is in the range of about 1 picogram to about 5 nanograms per cell. Effective doses and optimal dose ranges may be determined *in vitro* using the methods described herein.

The compounds of the invention may be characterized as producing an inhibitory effect on tumor cell or endothelial cell proliferation, migration, invasion, or on angiogenesis, on tumor metastasis or on inflammatory reactions. The compounds are especially useful in producing an anti-tumor effect in a mammalian host, preferably human, harboring a tumor.

Angiogenesis inhibitors may play a role in preventing inflammatory angiogenesis and gliosis following traumatic spinal cord injury, thereby promoting the reestablishment of neuronal connectivity (Wamil, A.W. *et al.*, *Proc. Nat'l. Acad. Sci. USA* 95:13188-13193 (1998)). Therefore, the compositions of the present invention are administered as soon as possible after traumatic spinal cord injury and for several days up to about two weeks thereafter to inhibit the angiogenesis and gliosis that would sterically prevent reestablishment of neuronal connectivity. The treatment reduces the area of damage at the site of spinal cord injury and facilitates regeneration of neuronal function and thereby prevents paralysis. The compounds of the invention are expected also to protect axons from Wallerian degeneration, reverse aminobutyrate-mediated depolarization (occurring in traumatized neurons), and improve recovery of neuronal conductivity of isolated central nervous system cells and tissue in culture.

GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd (or later) Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al.* *Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, editor, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd (or later) Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd (or later) Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd (or later) Ed., University of California Press, Berkeley, CA (1981).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompass conservative substitution variants thereof (*e.g.*, degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below).

ability of the HPRG polypeptide to inhibit angiogenesis, endothelial tube formation, cell invasion or tumor growth or metastasis.

Generally, the nucleic acid sequence encoding a fragment of HPRG comprises of nucleotides from the sequence encoding the mature protein (or the active H/P domain thereof).

5 Nucleic acid sequences, particularly those that encode peptide multimers of this invention may also include linker or spacer sequences (preferably encoding Gly₁₋₆). The nucleic acids further may include natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded polypeptide or peptides. These and other modifications of nucleic acid sequences are described herein or are well-known in
10 the art.

The techniques for assembling and expressing DNA coding sequences include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

15 EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a HPRG polypeptide, domain, peptide or peptide multimer operably linked to at least one regulatory sequence.

20 The term “expression vector” or “expression cassette” as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

25 “Operably linked” means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term “regulatory sequence” includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology*.
30 *Methods in Enzymology*, vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant “naked DNA” vector, and the like. A “vector” comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (*e.g.*, a cell membrane, a viral lipid envelope, *etc.*). Vectors include, but are not limited to replicons (*e.g.*, RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.*, plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is a host for an “expression vector,” this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host’s genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and the nature (*e.g.*, size) of the polypeptide to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the HPRG polypeptide, domain or peptide fragment and its including peptide multimers, variants, *etc.*

Such expression vectors are used to transfect host cells (*in vitro*, *ex vivo* or *in vivo*) for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the HPRG polypeptide, domain, peptide fragment or multimer, may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the HPRG polypeptide or H/P , domain and DNA encoding at least a portion of a second HPRG-derived sequence (or variant), so that the host cells produce yet further HPRG polypeptide, domain or peptide fragments that include both the portions.

Summers, M. D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM 8 (Aruffo A. and Seed, B., *supra*, for transient amplification/expression in mammalian cells, while CHO (*dhfr*-negative CHO) cells are used with vectors such as pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195) for stable
 5 amplification/expression in mammalian cells. The NS0 myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such
 10 cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant polypeptide.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by
 15 coexpressed viral RNA polymerase (T7gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.
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Vector Construction

Construction of suitable vectors containing the desired coding and control sequences
 25 employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired. The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence,
 30 initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to

synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tetrahed Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using well-known methods.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Meth Enzymol* (1980) 65:499-560.

Any of a number of methods are used to introduce mutations into the coding sequence to generate variants of the invention if these are to be produced recombinantly. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases. Modifications of the DNA sequence are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al.*, *Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al.*, *DNA* (1983) 2:183-193)). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al.*, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al.*, *Meth. Enzymol.*, *supra*.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts. In fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell* 41:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA* 79:6777 (1982)). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell* 31:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature* 290:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 81:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) 231:699; Fields *et*

al., *Nature* (1989) 340:245; Jones, *Cell* (1990) 61:9; Lewin, *Cell* (1990) 61:1161; Ptashne *et al.*, *Nature* (1990) 346:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No. 5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

DELIVERY OF NUCLEIC ACID TO CELLS AND ANIMALS

DNA delivery involves introduction of a "foreign" DNA either (1) into a cell *ex vivo* and ultimately, into a live animal by administering the cells, or (2) directly into the animal. Several general strategies for "gene delivery" (*i.e.*, delivery of any nucleic acid vector) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol.* 12:335-356 (1992); Anderson, W.F., *Science* 256:808-813 (1992); Miller, A.S., *Nature* 357:455-460 (1992); Crystal, R.G., *Amer. J. Med.* 92(suppl 6A):44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci.* 618:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol.* 38:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest.* 7:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

Preferred DNA molecules for delivery as described below encode HPRG, *e.g.*, SEQ ID NO:1 or 3, the H/P domain thereof (SEQ ID NO:5 or 6) or peptides or peptide multimers based on SEQ ID NO:7, 8, 9 or 10.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below.

These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay.

Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an indefinite period of time (Wolff, J.A. *et al.*, *Science* 247:1465 (1990); Acsadi, G. *et al.*, *The New Biologist* 3:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem.* 265:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy* 2:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science* 252:431 (1991)); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Manneheim1 Biochemicals, USA, 1991). Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

is also useful for human therapy (Samulski, R.J. *et al.*, *EMBO J.* 10:3941 (1991) in the present invention.

Another useful vector, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) 89:10847-10851; Fuerst, T.R. *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:2549-2553; Falkner F.G. *et al.*; *Nucl. Acids Res* (1987) 15:7192; Chakrabarti, S *et al.*, *Molec. Cell. Biol.* (1985) 5:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) 3:86-90; Moss, B. *Biotechnology* (1992) 20:345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) 158:25-38; Moss, B., *Science* (1991) 252:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* 291, 238-239 (1981); Poirier, TP *et al.* *J. Exp. Med.* 168, 25-32 (1988); (Sadoff, J.C., *et al.*, *Science* 240, 336-338 (1988); Stover, C.K., *et al.*, *Nature* 351, 456-460 (1991); Aldovini, A. *et al.*, *Nature* 351, 479-482 (1991); Schafer, R., *et al.*, *J. Immunol.* 149, 53-59 (1992); Ikonomidis, G. *et al.*, *J. Exp. Med.* 180, 2209-2218 (1994)). These organisms permit enteric routes of infection, providing the possibility of oral nucleic acid delivery.

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett.* 280:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett.* 244:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol.* 27:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules of the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta* 1088:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al.*, *J. Biol. Chem.* 264:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem.* 263:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA* 80:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem.* 267:963 (1992)). Preferred carriers are targeted liposomes

(Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA* 80:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (*e.g.*, asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA of the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Rabbit HPRG is cleaved by plasmin to release the His-Pro-rich domain (H/P) and the residual N/C domain. The domain structure is illustrated in Figure 1. The scissors in the Figure illustrate the positions of plasmin cleavage.

These domains can then be purified and tested *in vitro* and *in vivo* for anti-angiogenic activity in order to identify the region of HPRG that mediates anti-angiogenic effects.

EXAMPLE II

Inhibition of Endothelial Cell Proliferation by HPRG

bFGF is used to stimulate human umbilical vein endothelial cell (HUVEC) proliferation. Cells are incubated in the presence of bFGF alone or with added inhibitors of proliferation for 48 hours in a 96 well plate. Proliferation is then measured using the colorimetric reagent, MTS. Results in Figure 2A and 2B are presented as a percentage of the proliferation observed in wells incubated with bFGF alone (100% proliferation).

Rabbit HPRG (rHPRG) inhibited bFGF-stimulated proliferation of HUVEC in a dose dependent manner, the inhibition being almost complete at 100 nM as shown in Fig. 2A.

Fig. 2B shows that the H/P domain prepared by limited proteolysis of HPRG by plasmin retained the anti-proliferative activity of intact HPRG, whereas the proteolysis product (the N/C

domain, which included all the domains of HPRG but the H/P domain) had no activity. HKa is included as a positive control.

Viability of HUVEC treated with 1 μ M HPRG was not less than controls, indicating that this polypeptide is not cytotoxic to HUVEC.

5

EXAMPLE III

HPRG and the H/P Domains Induce Endothelial Cell Apoptosis Through the Induction of Caspase-3 Activity

10 In order to evaluate whether the observed anti-proliferative activity of HPRG was due to an induction of apoptosis, the activity of caspase-3 (an enzyme that is central to several pre-apoptotic pathways) was measured. HUVEC were grown in 100 mm² petri dishes in the presence of bFGF or bFGF + HPRG. Cells were extracted and caspase-3 activity measured using a fluorescent substrate.

15 HKa, which had previously been shown to induce caspase-3 activity in HUVECs [Zhang et al., FASEB J(2000) 14: 2589-2600] was used as a positive control.

The results are shown in Figure 3. rHPRG at 10 and 100 nM induced caspase-3 activity to a similar degree as did HKa. Results are expressed as percent of HKa activity, taken to be 100% in this assay). The H/P domain of HPRG also induced apoptosis to a similar extent at similar concentrations of H/P in the assay. This indicates that the apparent decrease in cell number
20 observed in the proliferation assay may be related to a direct induction of cell death in endothelial cells by HPRG and its H/P domain.

EXAMPLE IV

Rabbit HPRG Inhibits Endothelial Cell Tube Formation of HUVECs on Matrigel®

25 HUVEC were seeded onto Matrigel®-coated 96 well plates. Photomicrographs showing results are in Figure 4A and 4B.

Endothelial cell tube formation on Matrigel® was stimulated by incubation for 24 hr with FGF-2 (20 ng/ml), VEGF (20 ng/ml) and PMA (40 ng/ml) for 24 hours (Fig. 4A). Addition of HPRG (500 nM) almost completely disrupted tube formation under these conditions (Fig. 4B).

30

EXAMPLE V

HPRG (ATN-234) and the H/P (ATN-236) Domain Inhibit Angiogenesis in the CAM Model

This assay was performed essentially as described by Nguyen *et al.* (*Microvascular Res.* 47:31-40 (1994)). A filter containing either an angiogenic factor (bFGF, 30 µg/ml) or bFGF at the same concentration and an inhibitor at 20 µg/ml, was placed onto the CAM of an 8-day old chick embryo, and the CAM was observed for 3-9 days. Angiogenesis was quantitated by counting the number of microvessels that contacted the filter. In this experiment, microvessel were counted 4 days after implantation of the filter.

As shown in Figure 5 HPRG (ATN-234), HKa (ATN-235) and ATN-236 (H/P domain) were all capable of inhibiting neovessel formation in this model.

EXAMPLE VI

HPRG and the H/P Domain Inhibit Angiogenesis stimulated by FGF-2 in Matrigel® Plug model *in vivo*

In this study, ice-cold Matrigel® (500 µL) was mixed with heparin (50 µg/ml), FGF-2 (400 ng/ml) and the compound to be tested. The Matrigel® mixture was injected subcutaneously into 4-8 week-old female Ncr athymic nude mice at sites near the abdominal midline, 3 plugs per mouse. The injected Matrigel® forms a palpable solid gel. Animals were sacrificed by cervical dislocation 7 days post injection. The mouse skin was detached along the abdominal midline, and the Matrigel® plugs were recovered and scanned immediately at high resolution. Plugs were then dispersed in water and incubated at 37°C overnight. Hemoglobin levels were determined using Drabkin's solution (from Sigma) according to the manufacturers' instructions.

The results are shown in Figure 6. HPRG (0.25 µM, A) and the H/P domain (0.6 µM, C) completely inhibited angiogenesis. In contrast, the N/C fragment of HPRG (0.25 µM, C) had virtually no effect on angiogenesis. The Hb level compared to the positive control was 124 ± 42 % (mean \pm SD of three plugs).

EXAMPLE VII

HPRG and the H/P Domain Inhibit Tumor Cell (3LL)-Mediated Angiogenesis *In Vivo* in a Matrigel® Plug Model

The methods used in this study were essentially the same as described in Example VI except that 3LL tumor cells were used to stimulate angiogenesis instead of bFGF. Lewis lung

adenocarcinoma 3LL cells (10^6 cells/plug) were mixed with cold Matrigel prior to injection. After seven days, the animals are sacrificed by cervical dislocation and the Matrigel® plugs recovered and processed as above.

Results are shown in Figure 7 (where amount of Hb is shown). The control group of 3LL cells alone (A) shows a maximal level of angiogenesis, whereas, in the absence of tumor cells (B), a baseline of Hb presence is observed, reflecting control levels of vascularization. A “positive” control anti-angiogenic molecule, HKa (at 0.75 μM) (C) inhibits angiogenesis by about 50%. The H/P domain of HPRG (1.8 μM) (D) shows a similar degree of inhibition.

EXAMPLE VIII

HPRG and the H/P Domain Inhibit Tumor Cell (MatLyLu)-mediated Angiogenesis In Vivo in a Matrigel® Plug Model

The rat prostate tumor cell line (MatLyLu) was used to stimulate angiogenesis in the Matrigel® Plug model as described in Examples VII and VIII. In this study, tumor growth was evaluated.

Results are shown in Figure 8A and 8B. In the control group, plugs were inoculated with MatLyLu tumor cells alone. Introduction of the H/P domain (1.8 μ M) together with the tumor cells resulted in a significant diminution of tumor weight (Fig. 8A) and angiogenesis (Fig. 8B). Similar effects were observed with endostatin at the same concentration.

EXAMPLE IX

Identification of H/P consensus sequences

The H/P domain (was analyzed for the presence of repeat sequences. These are described below and the and quantitated in Table 1, below. Each consensus sequence has been compared for both rabbit and human sequences.

His-Pro domain of Human HPRG (residues 350-497) (SEQ ID NO:5)

350 360 370 380 390 400
H PHKHSHEQH PHGHHPHAHH PHEHDTHRQH PHGHHPHGHH PHGHHPHGHH
410 420 430 440 450
PHGHHPHCHD FQDYGPCDPP PHNQGHCCCHG HGPPPGHLRR RGP GK GPRPF
460 470 480 490 497
HCROIGSVYR LPPLRKGEVL PLPEANFPSP PLPHHKHPLK PDNQPF

His-Pro domain of Rabbit HPRG (residues 321-421) (SEQ ID NO:6)

5 321 330 340 350 360 370
 | | | | | |
 SVNIIHRPPP HGHHPHGPPP HGHHPHGPPP HGHPPHGPPP RHPPHGPPPH
 380 390 400 410 420
 | | | | |
 GHPPHGPPPH GHPPHGPPPH GHPPHGPPPH GHPPHGHGFH DHGPCDPPSH K

The sequences above are annotated to show three different consensus repeats:

HHPHG (in *italics*) - SEQ ID NO:8

10 **HPPHG** (in double underscore) - SEQ ID NO:9

PPPHG (in single underscore) - SEQ ID NO:10

This is shown below as a different version of SEQ ID NO:6

15 SVNIIHR PPPHG *HHPHG* PPPHG *HHPHG* PPPHG
 HPPHG PPPR HPPHG PPPHG HPPHG PPPHG
 HPPHG PPPHG HPPHG PPPHG HPPHG
 GFHDHGPCDPPSHK

TABLE 1

ATN# ¹	Repeated motif	SEQ ID NO:	# of repeats in	
			Rabbit	Human
ATN227	PPPHG	10	7	0
ATN228	HPPHG	9	6	0
ATN230	HHPHG	8	2	6

¹ Applicants' company designation of compound

20

EXAMPLE XI

Consensus Sequences from the HPRG H/P Domain Inhibit Angiogenesis

Matrigel® tube formation assays *in vitro* were carried out as described above.

25

Results are summarized in Table 2, below. Two consensus sequences from the H/P domain of HPRG, HHPHG (SEQ ID NO:8) and HPPHG (SEQ ID NO:9) were active in the Matrigel® Plug assay. The N-terminal Ala-substituted variant of the latter, APPHG (SEQ ID NO:11) had no effect on neovascularization as measured by tube formation in the Matrigel® assay.

TABLE 2

ATN#	Sequence	SEQ ID NO:	Inhibits Angiogenesis (Matrigel® assay)
ATN230	HHPHG	8	Yes
ATN228	HPPHG	9	Yes
ATN246	APPHG	11	No

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

- 5 Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.